F ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 November 1999 (25.11.99)	
International application No. PCT/FI99/00192	**Applicant's or agent's file reference**
International filing date (day/month/year) 15 March 1999 (15.03.99)	**Priority date** (day/month/year) 13 March 1998 (13.03.98)
Applicant HAKALEHTO, Eino, Elias	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 October 1999 (12.10.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer F. Baechler Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

2976969

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/FI99/00192	International filing date *(day/month/year)* 15.03.1999	Priority date *(day/month/year)* 13.03.1998
International Patent Classification (IPC) or national classification and IPC7 G 01 N 33/569		
Applicant Hakalehto, Elias		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12.10.1999	19.06.2000
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Telex 17978 PATOREG-S Facsimile No. 08-667 72 88	Authorized officer Carl-Olof Gustafsson/EE Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00192

I. Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

☐ the international application as originally filed.

☒ the description, pages 1-10, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☒ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-13, filed with the letter of 25-04-2000,
Nos. _____, filed with the letter of _____.

☒ the drawings, ~~sheets~~/fig 1-2, as originally filed,
~~sheets~~/fig _____, filed with the demand
~~sheets~~/fig _____, filed with the letter of _____,
~~sheets~~/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, ~~sheets~~/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00192

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-13	YES
	Claims		NO
Inventive step (IS)	Claims	5-7	YES
	Claims	1-4,8-13	NO
Industrial applicability (IA)	Claims	1-13	YES
	Claims		NO

2. Citations and explanations

The invention relates to a method for detecting bacteria in a cultivation medium prior to the peak of the population growth. In the method, bacterial antigens expressed by the cells soon after their inoculation to the enrichment medium, are detected. In preferred embodiments, the antigens are detected immunologically using antibodies. Furthermore, the bacteria preferably belong to the Salmonella genus.

The International Search Report revealed two documents of particular relevance:

A. GB 2234587 A, see especially example 2
B. WO 94/28420 A1, see abstract

Document A discloses a method in which E. coli is determined immunologically using ELISA after 5 hours of incubation at 37°C. From document A it is not clear whether or not the detection is performed "clearly prior to the peak of the population growth". However, the incubation time employed in document A is very similar to the one disclosed in claim 3. It is considered obvious to the person skilled in the art to use at least slightly shorter incubation times than those disclosed in document A. Thus, claims 1-4 and 8-12 are not considered to fulfil the requirement of inventive step in relation to document A.

Further, it can be noted that claim 1 does not provide any information regarding the cultivation conditions, such as temperature or medium, neither regarding which bacterium is being cultivated.

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

Since the method disclosed in document A has been applied to E. coli it is considered obvious to a person skilled in the art to use the same method for other bacteria, such as bacteria of the Salmonella genus, especially since document A also mentions Salmonella. Furthermore, minor variations of incubation temperatures corresponding to the optimum temperatures of selected bacteria, such as the use of 42°C instead of 37°C are considered obvious to the person skilled in the art. Therefore, claim 13 does not fulfil the requirement of inventive step.

Document B discloses the use of monoclonal antibodies in the immunological detection of bacterial antigens. It is considered obvious to a person skilled in the art to use monoclonal antibodies specific for bacterial proteins or peptides in the immunological determination disclosed in document A.

Claims 5-7 relate to the specific antigens detected. These claims are considered novel and are considered to involve an inventive step.

The invention as disclosed in claims 1-13 is considered industrially applicable.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00192

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 13 refers to "any of the claims 1-12" and discloses temperatures above 42°C. However, claim 12 refers to a temperature of about 37°C. Thus, claim 13 is contradictory. The claim has been interpreted as referring to "any of the claims 1-11".

25 -04- 2000

Amended Claims

1. A microbiological determination method, characterized in, that the bacteria are detected from their cultivation medium clearly prior to the peak of the population growth using the antigens which the cells express soon after their inoculation to the enrichment medium, before the actual growth phase or in the beginning of it.

2. A method according to the claim 1, characterized in, that the microbial antigens are detected immunologically using antibodies directly after the stationary phase.

3. A method according to the claim 2, characterized in, that the microbial antigens are detected immunologically in 3–4.5 hours after the onset of the enrichment culture.

4. A method according to any of the claims 2–3, characterized in, that the detected antigens are proteins.

5. A method according to any of the claims 1–4, characterized in, that the detected antigens are fimbrial proteins.

6. A method according to the claim 5, characterized in, that the detected antigens are type 1 fimbrial proteins or comparable to them.

7. A method according to any of the claims 1–6, characterized in, that the microbial antigens are detected with antibodies, which have been produced against the synthetic peptide Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Gln Val Pro Phe Ser Ile Val or a derivative thereof.

8. A method according to any of the claims 1–7, characterized in, that the detected microbes are enteric bacteria.

9. A method according to the claim 8, c h a r a c t e r i z e d in, that the detected microbes are fecal coliforms.

10. A method according to the claim 9, c h a r a c t e r i z e d in, that the detected microbes belong to genus *Salmonella*.

11. A method according to any of the claims 1–10, c h a r a c t e r i z e d in, that the microbes are incubated prior to the immunological detection in their optimal growth temperature.

12. A method according to the claim 11, c h a r a c t e r i z e d in, that the microbes are incubated prior to the detection at temperatures about 37 °C.

13. A method according to any of the claims 1–12, c h a r a c t e r i z e d in, that the microbes are incubated prior to the detection at temperatures above 42 °C.

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT / FI 99 / 00192

International Filing Date 15 MAR 1999 (15. 03. 99)

The Finnish Patent Office
PCT International Application
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)*

Box No. I TITLE OF INVENTION

METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

HAKALEHTO, Eino Elias
Kasarmikatu 12 C 1
FIN-70110 Kuopio
Finland

[X] This person is also inventor.

Telephone No. +358-17-240210

Facsimile No. +358-17-163919

Teleprinter No.

State *(that is, country)* of nationality: Finland

State *(that is, country)* of residence: Finland

This person is applicant for the purposes of: [X] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
[ ] applicant only
[ ] applicant and inventor
[ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

[ ] Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: [ ] agent [ ] common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Telephone No.

Facsimile No.

Teleprinter No.

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

See Notes to the request form

CONFIRMATION COPY

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP** **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

☒ **AL** Albania .....
☒ **AM** Armenia .....
☒ **AT** Austria .....
☒ **AU** Australia .....
☒ **AZ** Azerbaijan
☒ **BA** Bosnia and Herzegovina .....
☒ **BB** Barbados
☒ **BG** Bulgaria .....
☒ **BR** Brazil .....
☒ **BY** Belarus .....
☒ **CA** Canada
☒ **CH and LI** Switzerland and Liechtenstein
☒ **CN** China .....
☒ **CU** Cuba .....
☒ **CZ** Czech Republic .....
☒ **DE** Germany .....
☒ **DK** Denmark .....
☒ **EE** Estonia .....
☒ **ES** Spain .....
☒ **FI** Finland .....
☒ **GB** United Kingdom
☒ **GD** Grenada
☒ **GE** Georgia .....
☒ **GH** Ghana .....
☒ **GM** Gambia
☒ **HR** Croatia .....
☒ **HU** Hungary .....
☒ **ID** Indonesia
☒ **IL** Israel .....
☒ **IN** India .....
☒ **IS** Iceland
☒ **JP** Japan .....
☒ **KE** Kenya .....
☒ **KG** Kyrgyzstan .....
☒ **KP** Democratic People's Republic of Korea .....
☒ **KR** Republic of Korea .....
☒ **KZ** Kazakhstan .....
☒ **LC** Saint Lucia
☒ **LK** Sri Lanka
☒ **LR** Liberia
☒ **LS** Lesotho .....
☒ **LT** Lithuania
☒ **LU** Luxembourg
☒ **LV** Latvia
☒ **MD** Republic of Moldova .....
☒ **MG** Madagascar .....
☒ **MK** The former Yugoslav Republic of Macedonia .....
☒ **MN** Mongolia
☒ **MW** Malawi .....
☒ **MX** Mexico .....
☒ **NO** Norway
☒ **NZ** New Zealand .....
☒ **PL** Poland .....
☒ **PT** Portugal .....
☒ **RO** Romania
☒ **RU** Russian Federation .....
☒ **SD** Sudan
☒ **SE** Sweden
☒ **SG** Singapore
☒ **SI** Slovenia .....
☒ **SK** Slovakia .....
☒ **SL** Sierra Leone
☒ **TJ** Tajikistan .....
☒ **TM** Turkmenistan .....
☒ **TR** Turkey .....
☒ **TT** Trinidad and Tobago .....
☒ **UA** Ukraine .....
☒ **UG** Uganda .....
☒ **US** United States of America .....
☒ **UZ** Uzbekistan .....
☒ **VN** Viet Nam .....
☒ **YU** Yugoslavia .....
☒ **ZW** Zimbabwe .....

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

☒ .....
☒ .....
☒ .....

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

PCT/FI99/00192

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) 13 March1998 (13.3.1998)	980571	Finland		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): 1

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / SE

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*
Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	:	3
description (excluding sequence listing part)	:	11
claims	:	2
abstract	:	1
drawings	:	2
sequence listing part of description	:	
Total number of sheets	:	19

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ separate signed power of attorney
3. ☐ copy of general power of attorney; reference number, if any:
4. ☐ statement explaining lack of signature
5. ☐ priority document(s) identified in Box No. VI as item(s):
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☐ other *(specify)*:

Figure of the drawings which should accompany the abstract: 1

Language of filing of the international application: Finnish

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Elias Hakalehto

For receiving Office use only

1. Date of actual receipt of the purported international application: 15 MAR 1999 (15.03.99)
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority (if two or more are competent): ISA / SE
6. ☐ Transmittal of search copy delayed until search fee is paid.

For International Bureau use only

Date of receipt of the record copy by the International Bureau: 26 MARCH 1999 (26.03.99)

PCT/FI99/00192

MENETELMÄ MIKROBIEN OSOITTAMISEKSI RIKASTUSVILJELMÄSTÄ

Johdantoa

Salmonella on nykyään yksi tärkeimmistä elintarvikkeissa esiintyvistä bakteerikontaminanteista. Sille on ominaista nopea muuntautumiskyky, mikä aiheuttaa ongelmia sen osoitustehtävissä. Tällä hetkellä *Salmonella*-kantoja on karakterisoitu yli 2000, joista n. 100 on kliinisesti ja hygieenisesti merkittäviä. *Salmonella* on yleinen suolistosairauksien aiheuttaja ihmisille ja eläimille. Keskeisimpiä epidemioissa esiintyviä kantoja on noin kymmenen. Esiintyessään elintarvikkeissa *Salmonella* aiheuttaa usein laajojen ihmisjoukkojen altistumista infektiolle. Alkuperäisen kontaminaatiolähteen selvittäminen on haasteellinen tehtävä. Tavallinen tartunnantie kulkee saastuneiden elintarvikkeiden tai veden välityksellä. *Salmonella* kuuluu nk. enteerisiin balteereihin. Suurin osa kannoista aiheuttaa suolistotulehduksia.

Tavallisesti salmonellat ovat ihmisen tai isäntäeläimen elimistön ulkopuolella erittäin niukkojen kasvuolosuhteiden ympäristössä. Niiden on kyettävä säilymään elinkykyisinä esimerkiksi vedessä, missä niiden kanssa ravinteista kilpailee tavallisesti useita muita mikrobeja. Tällöin solut kehittyvät yleensä ilmeisesti eräänlaisiksi lepomuodoiksi. Näiden on hyviin kasvuolosuhteisiin päästyään kyettävä nopeasti muuntautumaan uusien olosuhteiden mukaisesti siten, että ne kykenevät kolonisoimaan esimerkiksi ruuansulatuskanavan pintaepiteelisolukkoa.

Elimistön ulkopuolella *Salmonella* ja muut enteeriset bakteerit ovat tavallisesti voimakkaan ympäristöstressin alaisia. Jouduttuaan ravinnon mukana elimistöön *Salmonella* ja muut bakteerit ja mikrobit joutuvat pian mahalaukun alhaisen pH:n ympäristöön, joka tuhoaa huomattavan osan elävistä mikrobisoluista. Alhainen pH toisaalta myös dissosioi solujen pinnalla olevia ulokkeita, joista erityisesti fimbriat tai vastaavat on tarkoitettu epiteeliin tarttumiseen. Siten mahalaukun jälkeen

pohjukaissuolessa salmonellojen ja muiden patogeenisten enteeristen lajien on elimistöön tunkeutuakseen nopeasti syntetisoitava erilaisten tarttumisrihmojen rakennusosat ja vastaavasti rakennettava nämä ulokkeet solujen pinnalle. Näitä tarttumisrihmoja voidaan toisaalta hyödyntää immunologisten mikrobiosoitusmenetelmien perustana, koska ne ovat yleensä voimakkaasti immunogeenisiä. Ne voidaan myös dissosioida rakennusosikseen, yksittäisiksi molekyyleiksi. Vastaavalla tavalla monien bakteerien liikkumistaan varten syntetisoimien flagellojen (uintisiimojen) ja niiden rakenneosien, flagelliiniproteiinien osoittamista voidaan hyödyntää näiden mikrobien immunologisessa osoittamisessa.

Tavallisesti ennen immunologista osoitusmenetelmää *Salmonella*-viljelmää tai -näytettä tai enteeristen bakteerien tai muiden bakteerien tai muiden mikrobien viljelmää joudutaan selektiivisesti kasvattamaan ja rikastamaan haluttuja mikrobeja.

Tavallisesti rikastusviljelyssä viljellään näytettä tai viljelmää niin kauan, että solujen lukumäärä moninkertaisesti ylittää alkuperäisen viljelmän näytemäärän, tavallisesti vähintään 12 tuntia. Yleisesti oletetaan, että tällöin myös osoitettavien antigeenien määrä lisääntyisi suunnilleen samassa suhteessa kuin solumäärä tai solumassa.

Salmonella-bakteerien leviämisen estäminen on keskeinen tavoite mm. elintarvike-, vesi- ja muussa ympäristöhygieniassa. Tästä syystä *Salmonella*n ja muiden vastaavien mikrobien osoittaminen on merkittävä ja laajeneva tutkimuksen ja taloudellisen toiminnan ala. Ongelmana *Salmonella*-bakteerien ja muiden taudinaiheuttajien diagnostiikassa on perinteisten bakteeriviljelymenetelmien vaatima pitkä aika ennen tuloksen saamista. Tämä aiheuttaa suuria kustannuksia esimerkiksi elintarviketeollisuudessa, jossa tuote-eriä joudutaan usein varastoimaan, kunnes hygieniakontrollin tulos on selvillä tai jos jo jakeluun saatettu tuote-erä joudutaan vetämään pois markkinoilta osoitustuloksen valmistuttua *Salmonella*n tai muun komtaminoivan bakteerin

toteamisen vuoksi. Mikrobimäärityksiin kohdistuvaa tutkimustoimintaa ja tuotekehittelyä onkin viime aikoina suunnattu nopeampien mikrobiosoitusmenetelmien löytämiseen.

Sairaaloissa kliinisten näytteiden ja esim. antibioottiresistenttien mikrobien hygieniakartoitusten tekemiseen tarvitaan nykyistä nopeampia, luotettavampia ja tehokkaampia menetelmiä, joiden olisi kuitenkin pystyttävä mikrobien osoittamiseen mahdollisimman yksinkertaisissa oloissa, jopa irti laboratorioista.

Myös elintarviketeollisuudessa tarvitaan tuoteturvallisuuden ylläpitämiseksi, varastointiaikojen lyhentämiseksi ja raaka-aine-erien tarkastamista varten uusia pikaosoitus- ja nopeita rikastusmenetelmiä. Samoin erilaisissa vesi- ja ympäristöanalyyseissä, joiden merkitys on viime aikoina korostunut, näiden menetelmien tarve on suuri.

Keksinnön taustaa

Osoitettaessa mikrobeja esimerkiksi kliinisistä näytteistä, elintarvike- tai ympäristönäytteistä, mikrobien pitoisuudet alkuperäisessä näytteessä ovat yleensä niin alhaiset, että tarvitaan nk. rikastusmenetelmiä, joiden avulla osoitettavien mikrobien määrää ja pitoisuutta näytteessä lisätään. Tähän käytetään eri mikrobeille soveltuvia spesifisiä viljelymenetelmiä, joihin sisältyy tavallisesti selektiivisen tekijän käyttö muiden mikrobien lisääntymisen estämiseksi. Tämä tekijä voi olla kemiallinen aine, antibiootti tai vastaava tai fysikaalinen tekijä kuten esimerkiksi kaasun osapaine. Myös pH voi olla selektiivinen tekijä. Usein voidaan selektiossa käyttää eri selektiivisten tekijöiden yhteisvaikutusta halutun mikrobin rikastusviljelyssä.

Mikrobien osoittamisessa rikastusmenetelmiin turvautuminen merkitsee ajanhukkaa ja siksi niiden toteuttamiseen tarvittavan ajan lyhentäminen on suotavaa.

Spesifisessä mikrobien tunnistamisessa käytetään usein eläimissä tai soluviljelmissä tuotettuja vasta-aineita (immunologiset menetelmät). Niitä käytetään usein mikrobien osoitukseen esimerkiksi rikastusviljelmistä. Ongelmana voi tällöin olla se, että testin suorittajalla ei ole tarkkaa tietoa siitä, sisältääkö tutkittava viljelmä tai muu näyte ja siinä olevat solut riittävästi osoitettavia antigeenisiä molekyylejä.

Keksintöä voidaan hyödyntää laajalti elintarvikkeissa esiintyvän *Salmonellan* monitoroinnissa. Esimerkiksi lihateollisuuden hygieniakontrollissa *Salmonella* esiintyy usein niin alhaisina pitoisuuksina, että sen suora osoittaminen immunologisella analyysillä on nykymenetelmillä mahdotonta. Tällöin tarvitaan monesti vähintään 24 tunnin rikastusviljely liemiputkissa. Rikastusviljely jakautuu usein kahteen vaiheeseen: esirikastukseen ja varsinaiseen rikastusviljelyyn. Esirikastuksen olosuhteita säätämällä voidaan myös nopeuttaa nimenomaan näytteessä mahdollisesti esiintyvien *Salmonella*-bakteerien kasvua ja sen haluttujen antigeenien ekspressoitumista. Kasvuolosuhteiden säätely auttaa myös sulkemaan pois ristireagoivia, osoitusta häiritseviä kantoja. Monien muiden bakteerien ja myös muiden mikrobien osoittamisessa ja identifioinnissa käytetään samalla tavalla apuna rikastusviljelyä.

Nopeuttaessaan *Salmonellan* ja muiden mikrobien osoitusta keksintö voi hyvin palvella esimerkiksi lihateollisuutta ja kliinistä diagnostiikkaa, joissa pikamenetelmiä kaivataan. Monesti teollisuuden piirissä tuotteiden säilyvyyden vuoksi niiden jakelu aloitetaan jo ennen mikrobiologisten monitorointitulosten valmistumista, mikä voi johtaa merkittäviin vahinkoihin kontaminaatioiden ilmetessä. Pikamenetelmät mahdollistaisivat pilaantuneiden erien poistamisen riittävän varhaisessa vaiheessa.

Keksinnön kuvaus

Kasvatettaessa *Salmonella*-suvun bakteerilajeja ja muita enteerisiä bakteereita selektiivisillä ravintoalustoilla havaittiin, että ne muodostivat runsaasti spesifisiä antigeenisiä molekyylejä jo ennen kuin varsinainen solujen lukumäärään perustuva mikrobipopulaation kasvu oli maksimissaan. Itse asiassa spesifisten vasta-aineiden havaitsemien antigeenien pitoisuus solujen pinnoilla oli oleellisesti vähentynyt huippulukemistaan solukasvun lähestyessä maksimiaan rikastusviljelmässä. Tämän vuoksi immunologisen osoitusmenetelmän käyttäminen voi tapahtua nykyisin tunnettuja menetelmiä aikaisemmin heti ns. stationäärisen vaiheen päättymisen jälkeen ennen kuin solujen lukumäärä on olennaisesti lisääntynyt esimerkiksi pesäkelaskuun perustuvien laskentamenetelmien avulla määritettäessä. Selektiivisenä tekijänä rikastusviljelyssä voidaan käyttää esimerkiksi lämpötilaa, ravintoalustan koostumusta, antibiootteja tai muita selektiivisiä molekyylejä sekä eri kaasujen osapaineiden säätelyä.

Tämän keksinnön mukaisella menetelmällä voidaan mikrobien identifioimista erilaisista näytteistä nopeuttaa käyttämällä hyväksi mikrobien erilaisten pintarakenteiden ekspression vaihtelua mikrobien kasvuvaiheen ja -olosuhteiden muutosten mukaan. Hyödyntämällä tätä "kiihdytettyä" antigeenien rikastamista voitiin esimerkiksi tyyppi 1 fimbria -antigeenien kohonneet pitoisuudet havaita 3 - 10 tuntia kuluttua viljelyn aloittamisesta (Suoritusesimerkit 4 - 5). Näitä mikrobien esimerkiksi ruoansulatuskanavan epiteelisolukkoon kiinnittymistä varten tuottamia fimbrioita tai niiden rakenneosia voidaan hyödyntää käytettyjä menetelmiä nopeammassa ? näiden mikrobien osoittamisessa.

Yhden mahdollisen soveltamismuodon puitteissa sopivan rikastusajan jälkeen mikrobisuspensiosta voidaan detektoida mikrobit hyödyntäen suodatinuuttoa (suomalainen patentti n:o 93742). Itse immunologinen osoitus voidaan toteuttaa immunoliuskalla, ELISA-menetelmällä, luminometrisella menetelmällä tai muulla vastaavalla laitteella tai menetelmällä käyttämällä uutetuille pintarakenteille

spesifisiä vasta-aineita. Tämä menettely voi teoriassa lisätä detektion herkkyyttä solumäärän ollessa vielä suhteellisen vähäinen.

Teoriassa mikrobien tarttumisominaisuuksia voitaisiin käyttää hyväksi myös näytteenottimena käytetyn injektioruiskun männän pinnoittamisessa (US. patentti n:o 5,846,209). Näytteenotossa käytetty mäntä voitaisiin vaihtaa esimerkiksi haetun bakteerin tarttumismolekyyleille spesifisillä vasta-aineilla tai tarttumismolekyylien kohteita matkivilla molekyyleillä päällystettyyn mäntään. Nestemäisiä näytteitä käsitellessä ei männän vaihto ole tarpeen. Sopiviin kasvuolosuhteisiin päästyään mikrobit alkavat ekspressoida tarttumismolekyylejään ja tarttuvat niillä männän pinnassa oleviin molekyyleihin. Kiinnittyminen todennetaan jollakin sopivalla menetelmällä (esimerkiksi sähköisesti tai optisesti).

Suoritusesimerkeissä 4 - 5 kuvataan eri *Salmonella*-kantoja vastaan tuotetun peptidivasta-aineen reaktioita eri vaiheissa olevien bakteeriviljelmien kanssa. Näissä kokeissa saatujen tulosten perusteella voitiin osoittaa korkeita immunologisia reaktiotasoja jo ennen varsinaisen logaritmisen kasvuvaiheen alkua. Tuotettaessa vasta-ainetta fimbria-proteiineille ja niistä saaduille peptideille todettiin, että voimakkaita immunologisia reaktiivisuuksia saatiin jo 3 - 5 tunnin kohdalla fimbria-peptideitä vastaan tehdyillä vasta-aineilla (Esimerkit 4 - 5). Tulokset viittaavat siihen, että kokeissa synteettisten peptidien tuottamisen lähdemateriaalina käytetty *Salmonella* tyyppi 1 fimbria -sekvenssi ekspressoituisi erityisen voimakkaasti jo ennen varsinaista logaritmista kasvuvaihetta tai heti sen alkaessa.

Tutkittaessa bakteeriviljelmien kasvua eri alustoilla ja eri kasvuolosuhteissa voitiin todeta, että esimerkiksi *S. enteritidis*-viljelmän kasvu selektiivisellä alustalla (RVS) oli voimakkaimmillaan +37 °C:ssa 3 - 6 tunnin kuluttua ja viljelmän massa lisääntyi lähelle maksimiaan 8 tunnissa (Esimerkki 4).

Teoreettisena perustana edellä kuvatulle ilmiölle on se, että joutuessaan ravinnon mukana suotuisaan ympäristöön ihmisen tai lämminverisen eläimen suolistoon *Salmonella*-bakteeri tai muu enteerinen bakteeri kasvattaa ensimmäiseksi fimbriat ja muut tarttumiseen tarvittavat molekyylirakenteet voidakseen kiinnittyä tähän suotuisaan ympäristöön, jossa ravinteita on runsaasti tarjolla. On myös mahdollista, että tarttumisproteiinit tai niiden prekursorit ovat ainakin osittain valmiina solulimassa tai vastaavassa paikassa jo lepovaiheen soluissa, mistä ne saadaan bakteerisolun kannalta mahdollisimman nopeasti käyttöön.

Suoritusesimerkki 1: *Salmonella*n kasvatus RVS-alustalla

Salmonella enteriditis -kantaa 9,12:-g, m:-, faagityyppi 4 (IHS 59813) ja *Salmonella typhimurium* -kantaa 4,5,12:i:1,2, faagityyppi 1 (IHS 59929) säilytettiin + 37° C:ssa THG -kasvatusliemessä (5% tryptoni, 2,5% hiivauute ja 1% glukoosi) ja nuorennettiin kahden viikon välein kokeen aikana. Käytetyt *Salmonella* -kannat oli saatu Kansanterveyslaitokselta (Helsinki, Suomi). Kasvatus aloitettiin 3 - 4 vuorokautta aiemin nuorennetuilla viljelmillä siirtämällä 5% viljelmää *Salmonella*lle selektiiviseen tuoreeseen RVS kasvualustaan (Rappaport-vassiliadis soija-peptoni-liemi, Oxoid, Englanti). RVS-suspensiota kasvatettiin ravistelluissa Erlenmeyer -pulloissa (kukin 100 ml) kahdessa lämpötilassa; + 37° C ja + 43° C. Näytteet otettiin kerran tunnissa.

Suoritusesimerkki 2: Peptidin kuvaus

Peptidisynteesin sekvenssi johdettiin *Salmonella typhimurium* tyyppi 1 fimbriasta. Jotta voitiin valita spesifinen sekvenssi, joka erosi vastaavasta *E.coli* tyyppi 1 fimbriasta, verrattiin kahta sekvenssiä. 18 aminohapon mittainen sekvenssi Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Glu Val Pro Phe Ser Ile Val valittiin. Peptidi oli syntesisoitu molekyyneinä, joissa 4 identtistä peptidiä oli liitetty toisesta päästään yhteen muodostaen näin multiple-antigen peptide'n (MAP). Peptidit syntesisoitiin Millipore PerSeptive 9050 Plus automaattisella

peptidisyntesoijalla ja Fmoc syntetisointi strategialla. Fmoc-Lys(Fmoc)-OH :ta käytettiin haaroitetun struktuurin runkona.

Suoritusesimerkki 3: Immunisoinnit

Peptidit käytettiin immunisointiin ilman sitomista kantajamolekyyleihin. Kanit immunisoitiin ruiskuttamalla ihonalaisesti 500 µg MAP-peptidiä. Immunisointiliuos sisälsi myös Freund's complete adjuvant'ia. Toistoimmunisoinnit tehtiin kahden viikon välein. Liuos sisälsi myös Freund's incomplete adjuvant'ia. Immunisointien yhteydessä otettiin kaneista seeruminäytteet, jotka talletettiin pakasteeseen (-20oC).

Suoritusesimerkki 4:

Peptidivasta-aineen reaktioiden tutkimiseksi eri kasvuvaiheessa olevien bakteeriviljelmien kanssa tehtiin kasvunopeus-ELISA -koe käyttäen kahta bakteeritiheydeltään erilaista bakteeriviljelmää. Kokeessa käytettiin *Salmonella entertidis* -kantaa IHS 59813, kasvualustana oli RVS-liemi ja kasvatuslämpötilana + 37 oC. ELISA-mittausten rinnalla tehtiin maljaviljelyt. Bakteeritiheydet olivat kokeen alkaessa 1,3 E+6 ja 1,0E+4. ELISA-kokeen ja maljaviljelyjen tulokset on esitetty taulukossa 1 ja kuvassa 1. Kasvunopeus-ELISA toteutettiin seuraavalla tavalla:

1. Käsiteltiin kuoppalevyt glutaraldehydillä:
a) Käytettäviin kuoppiin lisättiin 150 ug 0,5 % glutaraldehydiliuosta ja
b) inkuboitiin kuoppalevyä huoneenlämmössä tasoravistelijassa 15 minuuttia.

2. Pesut: Kaadettiin glutaraldehydi pois kuopista ja pestiin kuopat kaksi kertaa 200 ul:lla ELISA:n 1 x pesuliuosta (5mM Tris + 0,15 M NaCl + 0,05 Tween20).

3. Lisättiin haluttuihin kuoppiin antigeeniä 1:1 Assay Buffer-laimennoksena 50 ul/kuoppa (Assay Buffer PBS + 1%BSA + 0,05%NaN3 + 1mM EDTA).

Nollakuoppiin, joihin ei lisätty antigeenia, pipetoitiin 50 ul 1x pesuliuosta/kuoppa. Tämän jälkeen kuoppalevyä inkuboitiin folioon käärittynä kylmiössä yön yli.

4. Seuraavana päivänä kuopat kaadettiin tyhjiksi ja pestiin kolme kertaa kuten edellä.

5. Blokkausta varten kuoppiin lisättiin 1,5% BSA/TBS-liuosta 200 ul/kuoppa ja kuoppalevyä inkuboitiin tasoravistelijassa yksi tunti.

6. Kuopat pestiin kuten kohdassa 4.

7. Lisättiin primääriset vasta-aineet (peptidiseerumi H463):
a) Pipetoitiin seerumilaimennosta 1:100 50 ul/kuoppa.
b) Inkuboitiin levyä tasoravistelijassa huoneenlämmössä ½ tuntia.

8. Pestiin kuopat neljä kertaa kuten edellä.

9. Lisättiin sekundaariset vasta-aineet:
a) Pipetoitiin sekundaarivasta-aineen (anti-rabbit) laimennosta 1:1000 50 ul/kuoppa
b) Inkuboitiin levyä huoneenlämmössä tasoravistelijassa ½ tuntia.

10. Pestiin levyt kolme kertaa kuten edellä ja yhden kerran Afos Bufferilla (200 ul/kuoppa).

11. Väriliuosta (pNPP) pipetoitiin levylle 50 ul/kuoppa.

12. Absorbanssi mitattiin ELISA-readerilla (aallonpituus 405 nm) 15 minuutin, puolen tunnin, yhden tunnin, puolentoista tunnin ja kolmen tunnin kuluttua värin lisäämisestä.

Taulukko 1. Absorbanssilukemat IHS 59813-antigeenille (yhden tunnin kuluttua värin lisäämisestä) ja bakteeritiheydet ajan funktiona

Bakteeritiheys alussa $1,3*10^6$

Aika	0 h	2,5h	3,5h	4,5h	5,5h	6,5h	7,5h	8,5h	9,5h
0-absorbanssi	0,245	0,245	0,245	0,232	0,227	0,227	0,227	0,230	0,234
absorbanssilukema	0,228	0,233	0,248	0,464	0,248	0,483	0,437	0,345	0,376
bakteeritiheys	$1,3*10^6$	$9,7*10^5$		$1,8*10^6$		$7,7*10^8$		$1,3*10^9$	$1,8*10^9$

Bakteeritiheys alussa $1,0*10^4$

Aika	0 h	2,5h	3,5h	4,5h	5,5h	6,5h	7,5h	8,5h	9,5h
0-absorbanssi	0,222	0,226	0,227	0,227	0,227	0,227	0,225	0,225	0,225
absorbanssilukema	0,216	0,238	0,235	0,229	0,218	0,249	0,346	0,332	0,314
bakteeritiheys	$1,0*10^4$	$1,1*10^4$		$1,9*10^4$		$5,3*10^6$		$1,8*10^8$	$2,7*10^{10}$

Suoritusesimerkki 5:

Peptidivasta-aineen reaktioiden tutkimiseksi eri kasvuvaiheessa olevien bakteeriviljelmien kanssa tehtiin kasvunopeus-ELISA -koe käyttäen kahta *Salmonella* -kantaa (IHS 59813 ja IHS 59929). Kasvualustana oli RVS-liemi ja kasvatuslämpötilana 43 oC. ELISA- kokeen rinnalla tehtiin maljaviljelyt. Koe tehtiin kuten suoritusesimerkissä 4. Tulokset on esitetty taulukossa 2 ja kuvassa 2.

Taulukko 2. Absorbanssilukemat IHS 59813-antigeenille ja IHS 59929-antigeenille (kolmen tunnin kuluttua värin lisäämisestä) ja bakteeritiheydet ajan funktiona

Kanta IHS 59813

Aika	0 h	1h	2h	3h	4h	5h	6h	7h	8h
0-absorbanssi	0,346	0,346	0,346	0,336	0,323	0,323	0,323	0,357	0,355
absorbanssilukema	0,369	0,362	0,428	0,739	1,202	1,120	0,928	0,826	0,859
bakteeritiheys	$1,0*10^7$		$1,3*10^7$		$1,4*10^8$		$1,5*10^8$		$1,7*10^8$

Kanta IHS 59929

Aika	0 h	1h	2h	3h	4h	5h	6h	7h	8h
0-absorbanssi	0,360	0,350	0,344	0,344	0,344	0,367	0,355	0,355	0,355
absorbanssilukema	0,392	0,427	0,480	0,733	1,136	0,867	0,697	0,726	0,671
bakteeritiheys	$7,7*10^6$		$1,9*10^7$		$4,0*10^7$		$2,2*10^8$		$5,5*10^8$

Kirjallisuutta

Hakalehto, E. Suomalainen patentti n:o 93742, Menetelmä ja laite solujen osoittamiseksi, 1995.

Hakalehto, E. US. patentti n:o 5,846,209, Syringe comprising an adhering substrate for microbes, 1998

Vaatimukset

1. Mikrobiologinen määritysmenetelmä, t u n n e t t u siitä, että mikrobit osoitetaan kasvualustaltaan selvästi ennen populaation solukasvun huippua solujen pian rikastusalustaan siirrostamisen jälkeen ekspressoimien antigeenien avulla.

2. Patenttivaatimuksen 1 mukainen menetelmä, t u n n e t t u siitä, että mikrobiantigeenit osoitetaan immunologisesti vasta-aineiden avulla.

3. Patenttivaatimuksen 2 mukainen menetelmä, t u n n e t t u siitä, että mikrobiantigeenit osoitetaan immunologisesti alle 10 tunnin kuluttua rikastuskasvatuksen alettua.

4. Patenttivaatimuksen 2 mukainen menetelmä, t u n n e t t u siitä, että mikrobiantigeenit osoitetaan immunologisesti alle 6 tunnin kuluttua rikastuskasvatuksen alettua.

5. Jonkin patenttivaatimuksista 2 - 4 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat antigeenit ovat proteiineja.

6. Jonkin patenttivaatimuksista 2 - 5 mukainen menetelmä, t u n n e t t u siitä, että mikrobiantigeenit osoitetaan vasta-aineilla, jotka on tuotettu synteettisiä peptidejä vastaan.

7. Jonkin patenttivaatimuksista 1 - 6 mukainen menetelmä, t u n n e t t u siitä, että immunisointiin käytettävä peptidi on Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Glu Val Pro Phe Ser Ile Val tai sen johdannainen.

8. Jonkin patenttivaatimuksista 1 - 7 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat antigeenit ovat fimbria-proteiineja.

9. Jonkin patenttivaatimuksista 1 - 8 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat antigeenit ovat tyyppi 1 fimbria-proteiineja tai vastaavia.

10. Jonkin patenttivaatimuksista 1 - 9 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat mikrobit ovat bakteereja.

11. Jonkin patenttivaatimuksista 1 - 10 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat mikrobit ovat enteerisiä bakteereja.

12. Jonkin patenttivaatimuksista 1 - 11 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat mikrobit ovat fekaalisia koliformeja.

13. Jonkin patenttivaatimuksista 1 - 12 mukainen menetelmä, t u n n e t t u siitä, että osoitettavat mikrobit kuuluvat sukuun *Salmonella*.

14. Jonkin patenttivaatimuksista 1 - 13 mukainen menetelmä, t u n n e t t u siitä, että mikrobeja inkuboidaan ennen immunologista osoitusta niiden optimilämpötilassa.

15. Jonkin patenttivaatimuksista 1 - 13 mukainen menetelmä, t u n n e t t u siitä, että mikrobeja inkuboidaan ennen osoitusta noin 37° C lämpötilassa.

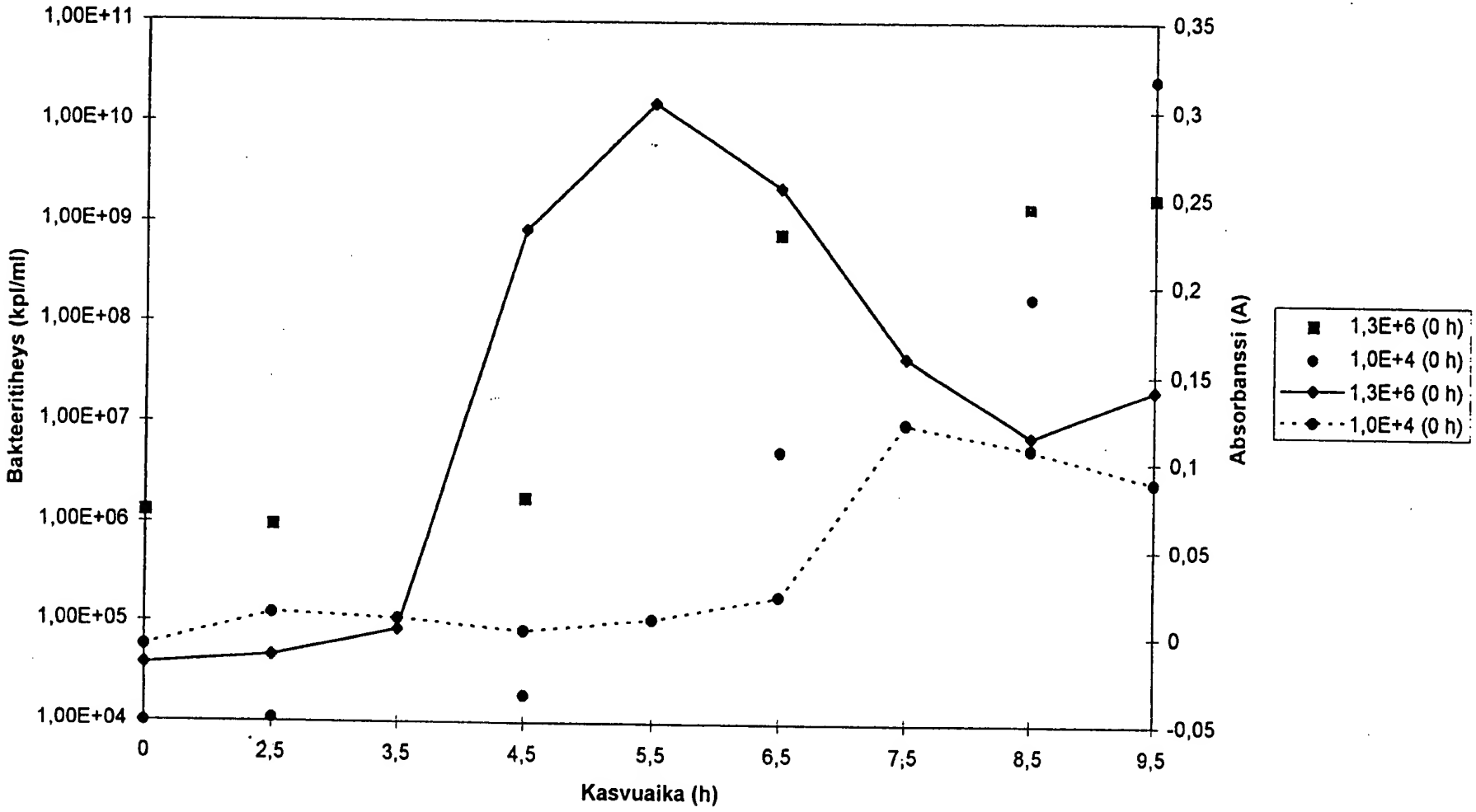
16. Jonkin patenttivaatimuksista 1 - 13 mukainen menetelmä, t u n n e t t u siitä, että mikrobeja inkuboidaan ennen osoitusta yli 40° C lämpötilassa.

PCT/FI99/00192

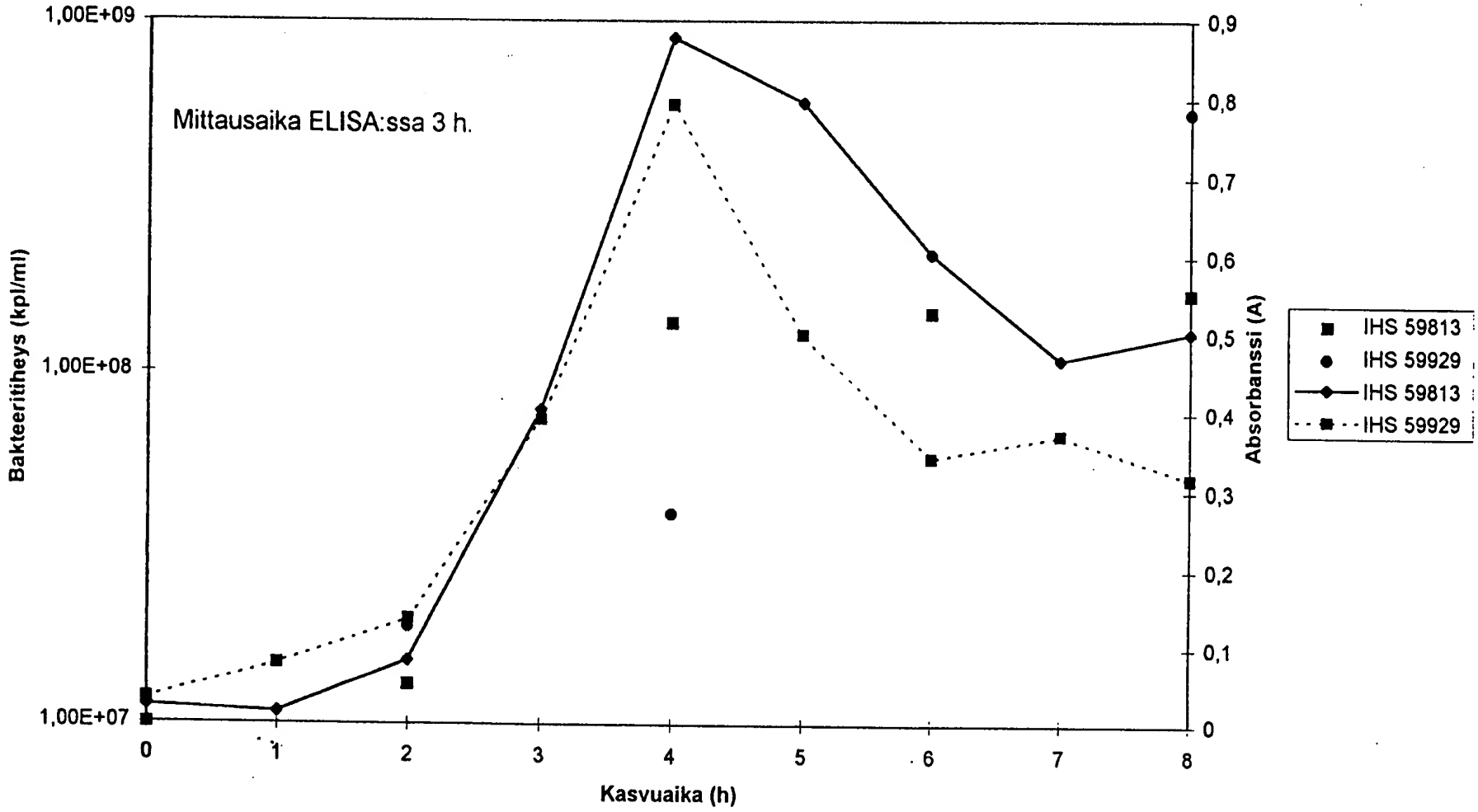
Tiivistelmä

Menetelmä mikrobien osoittamiseksi rikastuskasvatuksen aikaisessa vaiheessa näiden mikrobien jo ennen solukasvun logaritmista vaihetta aloittaman tiettyjen antigeenien voimakkaan ekspression avulla. Menetelmää voidaan hyödyntää erityisesti *Salmonella*n ja muiden enteeristen bakteerien osoittamiseen.

KUVA 1: **Kasvunopeus-ELISA 14.11.1997: IHS 59813 +37 oC:ssa, kun bakteeritiheys alussa 1,3E+6 ja 1,0E+4 kpl/ml sekä ks. kannan bakteeritiheys ajan funktiona.**



KUVA 2: **Kasvunopeus-ELISA 2.12.1997 sekä kasvu ajan funktiona 1.12.1997: kannat IHS 59813 ja IHS 59929 +43 oC:ssa. Primääriseerumina ELISA:ssa H463 1:100.**



PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/FI99/00192	International filing date *(day/month/year)* 15.03.1999	Priority date *(day/month/year)* 13.03.1998
International Patent Classification (IPC) or national classification and IPC7 G 01 N 33/569		
Applicant Hakalehto, Elias		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12.10.1999	19.06.2000
Name and mailing address of the IPEA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. 08-667 72 88 Telex 17979 PATOREG-S	Authorized officer Carl-Olof Gustafsson/EE Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00192

I Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

☐ the international application as originally filed.

☒ the description, pages 1-10, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☒ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-13, filed with the letter of 25-04-2000,
Nos. _____, filed with the letter of _____.

☒ the drawings, ~~sheets~~/fig 1-2, as originally filed,
~~sheets~~/fig _____, filed with the demand
~~sheets~~/fig _____, filed with the letter of _____,
~~sheets~~/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, ~~sheets~~/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00192

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	1-13	YES
	Claims		NO
Inventive step (IS)	Claims	5-7	YES
	Claims	1-4,8-13	NO
Industrial applicability (IA)	Claims	1-13	YES
	Claims		NO

2. Citations and explanations

The invention relates to a method for detecting bacteria in a cultivation medium prior to the peak of the population growth. In the method, bacterial antigens expressed by the cells soon after their inoculation to the enrichment medium, are detected. In preferred embodiments, the antigens are detected immunologically using antibodies. Furthermore, the bacteria preferably belong to the Salmonella genus.

The International Search Report revealed two documents of particular relevance:

A. GB 2234587 A, see especially example 2
B. WO 94/28420 A1, see abstract

Document A discloses a method in which E. coli is determined immunologically using ELISA after 5 hours of incubation at 37°C. From document A it is not clear whether or not the detection is performed "clearly prior to the peak of the population growth". However, the incubation time employed in document A is very similar to the one disclosed in claim 3. It is considered obvious to the person skilled in the art to use at least slightly shorter incubation times than those disclosed in document A. Thus, claims 1-4 and 8-12 are not considered to fulfil the requirement of inventive step in relation to document A.

Further, it can be noted that claim 1 does not provide any information regarding the cultivation conditions, such as temperature or medium, neither regarding which bacterium is being cultivated.

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

Since the method disclosed in document A has been applied to E. coli it is considered obvious to a person skilled in the art to use the same method for other bacteria, such as bacteria of the Salmonella genus, especially since document A also mentions Salmonella. Furthermore, minor variations of incubation temperatures corresponding to the optimum temperatures of selected bacteria, such as the use of 42°C instead of 37°C are considered obvious to the person skilled in the art. Therefore, claim 13 does not fulfil the requirement of inventive step.

Document B discloses the use of monoclonal antibodies in the immunological detection of bacterial antigens. It is considered obvious to a person skilled in the art to use monoclonal antibodies specific for bacterial proteins or peptides in the immunological determination disclosed in document A.

Claims 5-7 relate to the specific antigens detected. These claims are considered novel and are considered to involve an inventive step.

The invention as disclosed in claims 1-13 is considered industrially applicable.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/FI99/00192

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 13 refers to "any of the claims 1-12" and discloses temperatures above 42°C. However, claim 12 refers to a temperature of about 37°C. Thus, claim 13 is contradictory. The claim has been interpreted as referring to "any of the claims 1-11".

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/FI 99/00192	15 March 1999	13 March 1998
Applicant		
Hakalehto, Eino Elias		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] Certain claims were found unsearchable (See Box I).

2. [ ] Unity of invention is lacking (See Box II).

3. [X] The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing

   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] transcribed by this Authority.

4. With regard to the title,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the abstract,
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:
   Figure No. --
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI99/00192

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Due to lack of clarity (Art. 6 PCT) of the expression "antigens expressed by the cells" the search has been restricted to bacterial antigens.

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00192

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/569
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PATENT ABSTRACTS OF JAPAN, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2234587 A (CHISSO CORPORATION), 6 February 1991 (06.02.91), see especially example 2	1-4,10-16
Y	--	5-6
Y	WO 9428420 A1 (RHONE POULENC DIAGNOSTICS LTD), 8 December 1994 (08.12.94), see abstract	5-6
	--	

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1999	03 -07- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/ED Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/06/99

International application No.
PCT/FI 99/00192

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2234587 A	06/02/91	FR 2650673 A,B	08/02/91
		JP 3063571 A	19/03/91
WO 9428420 A1	08/12/94	NONE	

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
G01N 33/569

A1

(11) International Publication Number: WO 99/47931

(43) International Publication Date: 23 September 1999 (23.09.99)

(21) International Application Number: PCT/FI99/00192

(22) International Filing Date: 15 March 1999 (15.03.99)

(30) Priority Data:
980571 13 March 1998 (13.03.98) FI

(71)(72) Applicant and Inventor: HAKALEHTO, Eino, Elias [FI/FI]; Kasarmikatu 12 C 1, FIN–70110 Kuopio (FI).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
In English translation (filed in Finnish).

(54) Title: METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

(57) Abstract

A method for microbial detection in an early phase of the enrichment culture with the aid of extensive expression of certain antigens started by the microbes already before the logarithmic phase of the cell growth. The method can particularly be applied for the detection of *Salmonella* and other enteric bacteria.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

METHOD FOR DETECTING MICROBES FROM AN ENRICHMENT CULTURE

Introduction

Salmonella is nowadays one of the most important bacterial contaminants found in food products. Rapid adaptation capability, which is its typical feature, causes difficulties in detecting the bacterium. At present there are over 2000 characterized *Salmonella* strains of which about 100 are clinically and hygienically important. *Salmonella* is a common cause of enteric diseases for both humans and animals. The number of most important strains found during epidemics is about ten. The occurance of *Salmonella* in food often causes large amounts of people to get exposed to an infection. Finding the original source of contamination is a challenging task. Contaminated food or water provide a typical way for the contagion to spread. *Salmonella* belongs to the so called enteric bacteria. Most of the strains cause gastroenteritis.

Normally *Salmonellas* live outside the body of people or host animals in very poor growth conditions. They have to survive and retain their viability e.g. in water, where usually several other microbes compete with them for nutrients. Usually in these circumstances the cells probably develop into some form of resting cells. Once they reach good growth conditions they have to adapt quickly to the new conditions to be able to colonize e.g. the surface epithelial cells of the gastrointestinal tract.

Outside the body *Salmonella* and other enteric bacteria are usually under strong environmental stress. When the *Salmonella* or other micro-organisms get into the body with food, they soon enter the low-pH environment of the stomach, which destroys a large amount of living microbial cells. On the other hand, a low pH also dissociates appendiges on the cell surfaces, of which especially the fimbrias or corresponding structures are used for attaching onto the epithelium. Then after entering the duodenum the *Salmonellas* and other pathogenic enteric species in order to invade the body have to synthesize rapidly the building parts of the different attachment filaments and correspondingly build these appendiges on the cell surfaces. These filaments can be utilised as the basis of immunological methods for detecting microbes, because they are normally strongly immunogenic. They can also be dissociated into single molecules which are their building parts. In the same manner, we can make good use of the detection of the flagellas

that many bacteria synthesize to enable them to move and the flagellin protein, the building parts of the flagellum, in the immunological detection of microbes.

Normally, before using an immunological detection method, the *Salmonella* culture or sample or the culture of enteric bacteria, other bacteria or other micro-organisms must be selectively grown and the desired microbes enriched.

Normally in an enrichment cultivation the sample or culture is usually grown long enough for the number of cells to manifoldly outgrow that of the original sample, usually for at least 12 hours. It is generally supposed that also the amount of antigens increase in about direct ratio to the amount of cells or cell mass.

One of the key goals of food, water and other environmental hygiene is to prevent *Salmonella* bacteria from spreading. For this reason, the detection of *Salmonella* and other similar microbes is an important and broadening field of research and economic activity. The problem in using the traditional bacterial cultivation methods for diagnostics of the *Salmonella* bacteria and other pathogens is the long time needed to attain the results. This causes great expenses e.g. in food industry, where the products often need to be stored up while waiting for the results of the hygiene control or withdrawn from the market or distribution, if the results show contamination with *Salmonella* or other bacteria. Research and development work directed to microbial determinations has recently concentrated on finding more rapid detection methods for microbes.

For clinical sampling in hospitals and for the hygiene mapping of antibiotic resistant microbial strains, more rapid, more reliable and more effective methods are required. These methods should at the same time be useful also for detecting microbes in as simple conditions as possible, even outside laboratories.

Food industry also needs new rapid detection methods and fast methods of enrichment to maintain product safety, shorten storage time and control raw materials. Likewise, different water and environmental analysis, the significance of which has recently grown, need these methods urgently.

Background of the Invention

When detecting microbes from e.g. clinical samples, food samples or environmental samples the microbial concentrations in the original sample are usually so low that so called enrichment methods are needed. These methods increase the amount and concentration of the detected mibrobes in the sample. Microbe specific cultivation methods, which usually involve the use of a selective factor to prevent the multiplication of other microbes, are used. This factor can be a chemical substance, an antibiotic or an equivalent or some physical factor such as the partial pressure of a gas. The pH can also be a selective factor. Often the synergy of the different selective factors can be used in the enrichment culture of the desired microbe in a selection.

The need of having to use enrichment methods in detecting microbes means a loss of time and therefore the shortening of the time used for these procedures is most desirable.

The specific microbial identification often uses antibodies that are produced in animals or cell cultures (immunological methods). They are often used to detect microbes from enrichment cultures, for example. In such a case, the problem may be that the user of the test does not know for sure, if the culture in concern or other sample and the cells in it have enough antigenic molecules for the detection.

The invention can be applied in large scale for monitoring *Salmonella* in foodstuffs. For example in hygiene controls in the meat industry *Salmonella* often exists in so low percentages that the direct detecting using the immunological analysis is with present methods impossible. In these cases an enrichment culture in liquid medium for at least 24 hours is often needed. The enrichment process often divides into two phases: the pre-enrichment stage and the actual enrichment stage. By controlling the conditions of the pre-enrichment growth of the possible *Salmonella* bacteria and the expression of its desired antigens in the sample can be speeded up. Adjusting the conditions of the cultivation also helps to exclude the possibility of cross-reacting, disturbing strains.The enrichment culture is used in the same way as a help to detect and identify many other bacteria and also micro-organisms.

Because the invention makes the process of detecting *Salmonella* and other microbes faster, it can well serve e.g. meat industry and clinical diagnostics, which are in need of rapid methods. In industry the delivery of the products is often put into practice before the microbiological monitoring results are complete. This can lead into major losses if contamination should occur. The rapid methods would make possible to remove the spoiled products early enough.

Description of the Invention.

When growing bacteria species of the genus *Salmonella* and other enteric bacteria on selective nutrient media we noticed that they produced lots of specific antigenic molecules already before the actual microbial population growth, based on the number of cells, was at its maximum. In fact, the concentration of antigen that the specific antibody could detect on the cell surfaces had substantially lowered from its record figures as the cell growth in the enrichment culture grew near its maximum. For this reason the use of an immunological detection method can take place earlier than in the presently known methods, directly following the so called stationary phase before the number of cells, defined with e.g. the calculation methods based on the colony count, has markedly increased. For example, the temperature, the composition of the nutrient medium, antibodies or other selective molecules and the control of partial pressures of various gases can be used as selective factors in the enrichment culture.

According to the method of this invention, the identification process of microbes from various samples can be made more rapid by making use of the changes in the expressions of the different surface structures of the microbes according to the changes in the growth phase and growth conditions. By exploiting the "enhanced" enrichment of antigens we could e.g. observe the increased concentrations of type 1 fimbrial antigens in 3 – 10 hours from the onset of the cultivation (Examples 4 – 5). These fimbrias or their building parts, which the microbes produce e.g. to enable them to attach to the epithelial cells of the alimentary tract, can be made use of in methods for detecting microbes more rapidly than in the methods according to the present knowledge.

In one possible form of application, after a suitable period of enrichment it is possible to detect the microbes by taking advantage of filter extraction (Finnish patent No. 93742). The

immunological detection itself can be carried out with an immunostrip, the ELISA-method, a luminometric method or other corresponding apparatus or method using antibodies, which are suitable specifically for extracted surface structures. This procedure may, in theory, add the sensitivity of the detection when the amount of cells is still relatively small.

In theory, the attachment characteristics could be made use of also in coating the plunger of the injection syringe used in taking the sample (US. Patent No. 5,846,209). The plunger used in taking the sample could then be changed into a plunger coated with specific antibodies against to the attachment molecules or molecules that imitate the object of the attachment molecules of the searched bacteria. When handling fluid samples the plunger need not be changed. Once the microbes are brought into suitable growth conditions they begin to express their attachment molecules and attach with them to the molecules on the surface of the plunger. The attachment is verified with some suitable method (e.g. electrically or optically).

Examples 4 – 5 describe the reactions of a peptide antibody, produced against different *Salmonella* strains, with bacterial cultures in different growth phases. On the basis of the results from these experiments we were able to demonstrate high immunological reaction levels already before the beginning of the actual logarithmic growth phase. When producing antibody to the fimbrial proteins and to peptides derived from them, we noticed strong immunological reactivities already in 3 – 5 hours with antibodies produced against fimbrial peptides (Examples 4 – 5). The results implicate that the *Salmonella* type 1 fimbrial sequence, used in the experiment as source material in the production of synthetic peptides, would express outstandingly strongly already before the actual logarithmic growth phase or immediately at its beginning.

When examining the growth of the bacterial cultures on different media and in different growth conditions, we noticed that e.g. the growth of a *S. enteritidis*-culture in a selective medium (RVS) was at its strongest after 3 – 6 hours the temperature being +37°C and the mass of the cultivation grew close to its maximum in 8 hours (Example 4).

The basis of the phenomenon described above is the theory that reaching favourable conditions in the intestines of man or a warm-blooded animal the food-borne *Salmonella* bacterium or other enteric bacterium firstly produces fimbrias and other molecular structures required for attachment, in order to be able to stick to this favourable environment, where nutrients are

readily available. It is also possible that already in the stationary phase cells the attachment proteins or their precursors are at least partially prepared in the cytoplasm or in a corresponding site from where they can be mobilized in the fastest way from the point of view of the bacterial cell.

Example 1: Cultivation of *Salmonella* on RVS medium

Salmonella enteriditis strain 9,12:-g, m:-, phage type 4 (IHS 59813) and *Salmonella typhimurium* strain 4,5,12:i:1,2, phage type 1 (IHS 59929) were kept at 37 °C in THG medium (5% tryptone, 2.5% yeast extract and 1% glucose) and seeded in the intervals of two weeks during the experiment. The *Salmonella* strains were obtained from the National Public Health Institute (Helsinki, Finland). Cultivation was started from 3 – 4 days old culture by inoculating 5% of the culture to a fresh RVS medium (Rappaport-vassiliadis soya peptone broth, Oxoid, England), which is selective for *Salmonella*. The RVS suspension was cultivated in shaked Erlenmeyer flasks (each 100 ml) at two different temperatures: +37 °C and +43 °C. Samples were taken every hour.

Example 2: Description of the peptide

The sequence in the peptide synthesis was traced from the *Salmonella typhimurium* type 1 fimbriae. In order to select a specific sequence differing from the corresponding *E.coli* type 1 fimbriae, the two sequences were compared with each other. The sequence of 18 amino acids Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Gln Val Pro Phe Ser Ile Val was selected. The peptide was synthetized as molecules, in which 4 identical peptides were joined together from one end forming thus a multiple-antigen peptide (MAP). The peptides were synthetized with Millipore PerSeptive 9050 Plus automated peptide synthetizer and with Fmoc synthesis strategy. Fmoc-Lys(Fmoc)-OH was used as the backbone for the branched structure.

Example 3: Immunizations

Peptides were used for immunization without any conjugation to carrier molecules. Rabbits were immunized with 500 µg of MAP-peptide injected subcataneously. The immunization solution contained also Freund´s complete adjuvant. The boosters were injected in intervals of two weeks.

The solution contained also Freund´s incomplete adjuvant. Together with the immunizations the rabbits were bleeded in order to take serum samples, which were stored in the freezer (-20 °C).

Example 4:

For studying the reactions of the anti-peptide antibody against bacterial cultures in different growth phases, a "growth speed-ELISA" experiment was carried out using two bacterial cultures differing from each other in cell density. *Salmonella enteriditis* strain IHS 59813 was used in the experiment, the growth medium was RVS broth and the temperature +37 °C. Plate cultures were started simultaneously with ELISA meassurements. The bacterial densities were in the beginning of the experiment 1.3 E+6 and 1.0 E+4. Results from the ELISA experiment and the plate counts are presented in Table 1 and in Figure 1. "Growth speed ELISA" was carried out as follows:

1. The microtiter plates were treated with glutaraldehyde:
   a) 150 µg of 0,5% glutaraldehyde was added to the wells and
   b) the microplate was incubated at room temperature in a shaker for 15 minutes.
2. Washes: The glutaraldehyde was poured from the wells and the wells were washed twice with 200 µl of 1 x ELISA washing solution (5mM Tris + 0.15 M NaCl + 0.05 Tween20).
3. The antigen was added to the desired wells as an Assay Buffer dilution (1:1) 50 µl per well (Assay Buffer PBS + 1%BSA + 0.05%NaCL + 1mM EDTA).
   Into the zero wells, where no antigen was added, 50 µl 1 x washing solution was pipetted per well. Thenafter, the microplate was wrapped in a folio and incubated overnight in a cold room.
4. On the following day the wells were poured empty and washed three times as described above.
5. For blocking 1.5% BSA/TBS solution was added to the wells (200µl/well) and the microplate was incubated on a shaker for one hour.
6. The wells were washed as in 4.
7. The primary antibodies were added (peptide serum H463):
   a) Serum dilution (1:100) was pipetted (50 µl per well).
   b) The plate was incubated on a shaker for 0.5 hours at room temperature.
8. The wells were washed four times as above.

9. The secondary antibodies were added:

a) Secondary antibody (anti-rabbit) dilution 1:1000 was pipetted 50 µl per well.

b) The plate was incubated on a shaker for 0.5 hours at room temperature.

10. The plates were washed three times as above and once with Afos Buffer (200 µl/well).

11. Colour solution (pNPP) was pipetted (50 µl/well) to the plate.

12. Absorbance was measured with an ELISA-reader (wave length 405 nm) after 15 minutes, half an hour, one and a half hours and three hours after the addition of colour.

Table 1. The absorbance figures for IHS 59813 antigen (one hour after the addition of colour) and bacterial densities as the function of time.

Bacterial density in the beginning $1.3*10^6$

Time	0 h	2.5 h	3.5 h	4.5 h	5.5 h	6.5 h	7.5 h	8.5 h	9.5 h
0-absorbance	0.245	0.245	0.245	0.232	0.227	0.227	0.227	0.230	0.234
Absorbance	0.228	0.233	0.248	0.464	0.531	0.483	0.387	0.345	0.376
Bacterial density	$1.3*10^6$	$9.7*10^5$		$1.8*10^6$		$7.7*10^8$		$1.3*10^9$	$1.8*10^9$

Bacterial density in the beginning $1.0*10^4$

Time	0 h	2.5 h	3.5 h	4.5 h	5.5 h	6.5 h	7.5 h	8.5 h	9.5 h
0-absorbance	0.222	0.226	0.227	O.227	0.227	O.227	0.225	0.225	0.225
Absorbance	0.216	0.238	0.235	0.229	0.218	0.249	0.346	0.332	0.314
Bacterial density	$1.0*10^4$	$1.1*10^4$		$1.9*10^4$		$5.3*10^6$		$1.8*10^8$	$2.7*10^{10}$

Example 5:

For studying the reactions of the anti-peptide antibody against bacterial cultures in different growth phases, a "growth speed-ELISA" experiment was carried out using two different *Salmonella* strains (IHS 59813 ans IHS 59929). The growth medium was RVS broth and the cultivation temperature 43 °C . Plate cultures were started simultaneously with the ELISA experiment. The experiment was carried out as described in the Example 4. The results are presented in the Table 2 and Figure 2.

Table 2. The absorbance figures for IHS 59813 antigen and IHS 59929 antigen (three hours after the addition of colour) and bacterial densities as the function of time.

Salmonella strain IHS 59813

Time	0 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
0-absorbance	0.346	0.346	0.346	0.336	0.323	0.323	0.323	0.357	0.355
Absorbance	0.369	0.362	0.428	0.739	1.202	1.120	0.928	0.826	0.859
Bacterial density	$1.0*10^7$		$1.3*10^8$		$1.4*10^8$		$1.5*10^8$		$1.7*10^8$

Salmonella strain IHS 59929

Time	0 h	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
0-absorbance	0.360	0.350	0.344	0.344	0.344	0.367	0.355	0.355	0.355
Absorbance	0.392	0.427	0.480	0.733	1.136	0.867	0.697	0.726	0.671
Bacterial density	$7.7*10^6$		$1.9*10^7$		$4.0*10^7$		$2.2*10^8$		$5.5*10^8$

Literature

Hakalehto, E. Finnish patend n:o 93742, Menetelmä ja laite solujen osoittamiseksi, (A method and an apparatus for detecting cells), 1995.

Hakalehto, E. US. Patent n:o 5,846,209, Syringe comprising an adhering substrate for microbes, 1998.

Claims

1. A microbiological determination method, characterized in, that the microbes are detected from their cultivation medium clearly prior to the peak of the population cell growth using the antigens which the cells express soon after their inoculation to the enrichment medium.

2. A method according to the claim 1, characterized in, that the microbial antigens are detected immunologically using antibodies.

3. A method according to the claim 2, characterized in, that the microbial antigens are detected immunologically in less than 10 hours after the onset of the enrichment culture.

4. A method according to the claim 2, characterized in, that the microbial antigens are detected immunologically in less than six hours after the onset of the enrichment culture.

5. A method according to one of the claims 2-4, characterized in, that the detected antigens are proteins.

6. A method according to one of the claims 2-5, characterized in, that the microbial antigens are detected with antibodies, which have been produced against synthetic peptides.

7. A method according to one of the claims 1-6, characterized in, that the peptide used for immunization is Ala Ser Phe Thr Ala Ile Gly Asp Thr Thr Ala Gln Val Pro Phe Ser Ile Val or a derivative thereof.

8. A method according to one of the claims 1-7, characterized in, that the detected antigens are fimbrial proteins.

9. A method according to one of the claims 1-8, characterized in, that the detected antigens are type I fimbrial proteins or comparable to them.

10. A method according to one of the claims 1-9, c h a r a c t e r i z e d in, that the detected microbes are bacteria.

11. A method according to one of the claims 1-10, c h a r a c t e r i z e d in, that the detected microbes are enteric bacteria.

12. A method according to one of the claims 1-11, c h a r a c t e r i z e d in, that the detected microbes are fecal coliforms.

13. A method according to one of the claims 1-12, c h a r a c t e r i z e d in, that the detected microbes belong to genus *Salmonella*.

14. A method according to one of the claims 1-13, c h a r a c t e r i z e d in, that the microbes are incubated prior to the immunological detection in their optimal growth temparature.

15. A method according to one of the claims 1-13, c h a r a c t e r i z e d in, that the microbes are incubated prior to the detection at temperatures about 37 °C.

16. A method according to one of the claims 1-13, c h a r a c t e r i z e d in, that the microbes are incubated prior to the detection at temperatures above 40 °C.

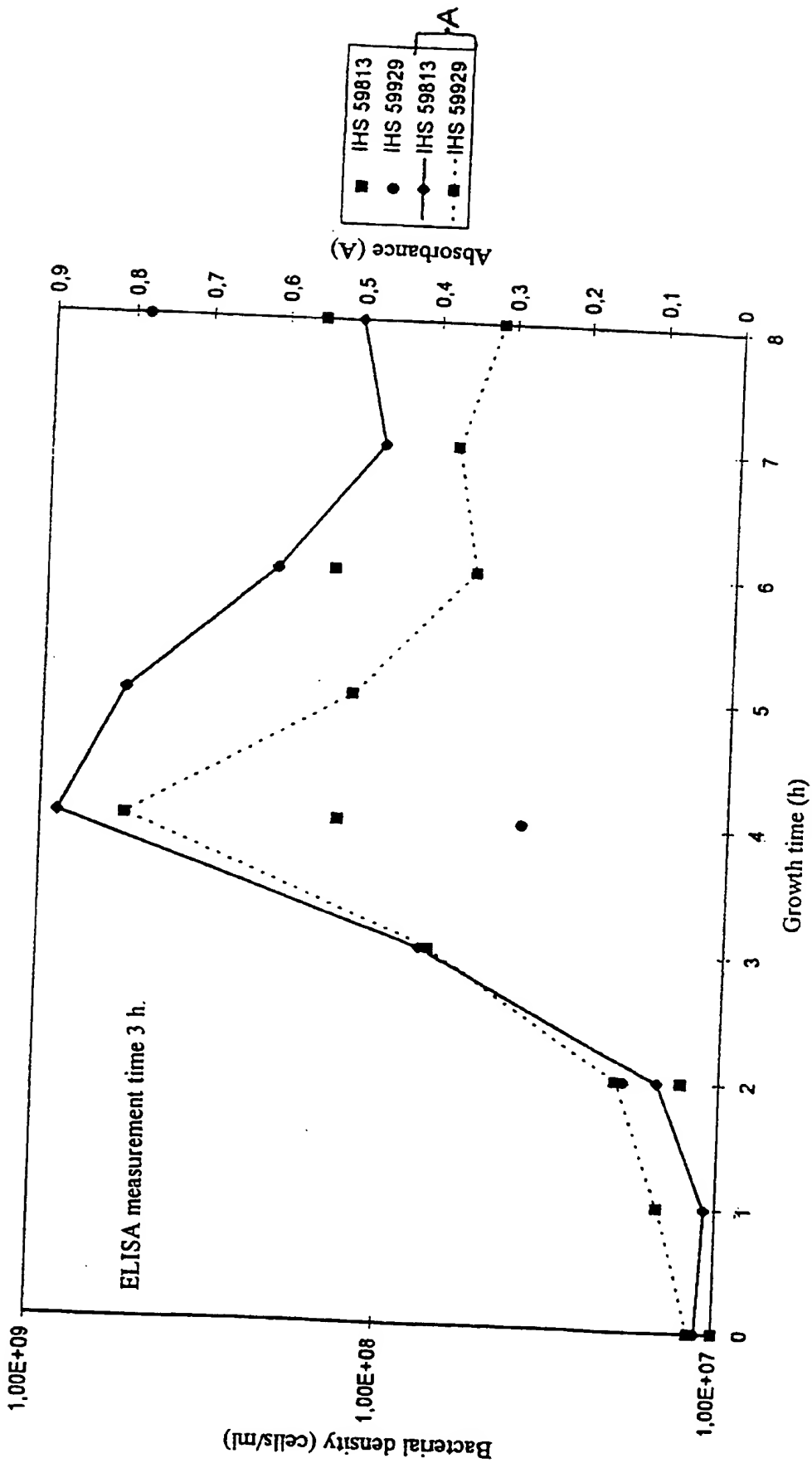


Figure 2. "Growth speed ELISA" 2.12.1997 and growth as a function of time 1.12.1997: strains IHS 59813 and IHS 59929 at +43 °C. Primary serum in ELISA H463 1:100.

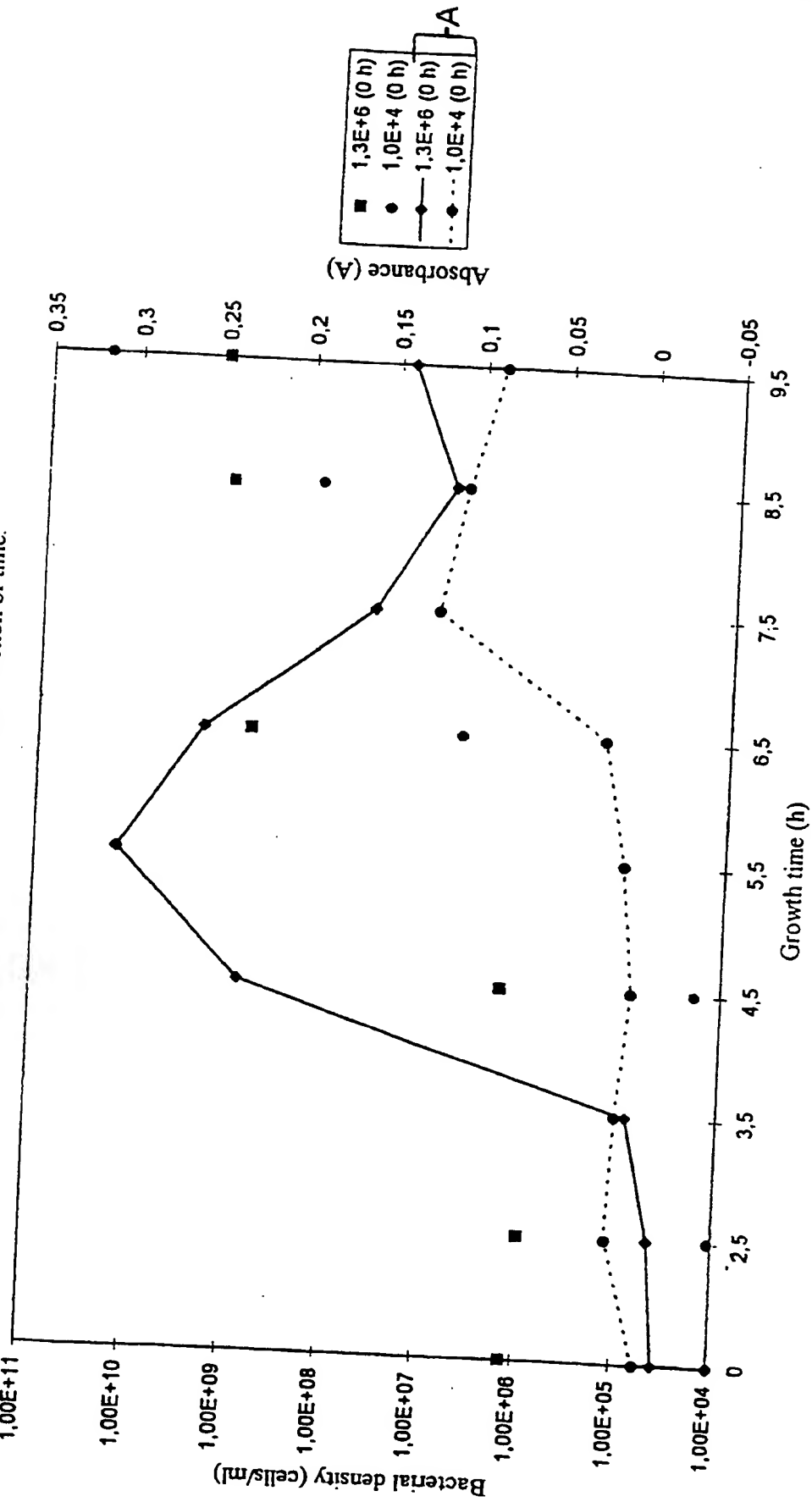


Figure 1. "Growth speed ELISA" 14.11.1997: IHS 59813 at +37 °C, bacterial density was in the beginning 1.3E+6 and 1.0E+4 cells/ml and bacterial density of the strain as a function of time.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 99/00192

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/569
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PATENT ABSTRACTS OF JAPAN, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2234587 A (CHISSO CORPORATION), 6 February 1991 (06.02.91), see especially example 2	1-4,10-16
Y	--	5-6
Y	WO 9428420 A1 (RHONE POULENC DIAGNOSTICS LTD), 8 December 1994 (08.12.94), see abstract	5-6
	--	

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1999	03 -07- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson/EÖ Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI99/00192

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Due to lack of clarity (Art. 6 PCT) of the expression "antigens expressed by the cells" the search has been restricted to bacterial antigens.

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/FI 99/00192

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2234587 A	06/02/91	FR 2650673 A,B	08/02/91
		JP 3063571 A	19/03/91
WO 9428420 A1	08/12/94	NONE	

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, G01N 33/569 — A1

(11) International Publication Number: WO 93/20231

(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/GB93/00647

(22) International Filing Date: 29 March 1993 (29.03.93)

(30) Priority data:
9207069.7 31 March 1992 (31.03.92) GB

(71) Applicant *(for all designated States except US)*: THE MINISTER OF AGRICULTURE, FISHERIES AND FOOD IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Whitehall Place, London SW1A 2HH (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WOODWARD, Martin, John [GB/GB]; 23 Burnsall Close, Farnborough, Hampshire GU14 8NN (GB). THORNS, Christopher, John [GB/GB]; 11 Lincoln Drive, Pyrford, Woking, Surrey GU22 8RL (GB).

(74) Agent: LOCKWOOD, Peter, Brian; DIPR IPR 1, Room 2002, Empress State Building, Lillie Road, London SW6 1TR (GB).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD OF TESTING FOR SALMONELLA

(57) Abstract

This invention relates to a method of testing for microorganisms of Salmonella serotypes, particularly *Salmonella Typhi (S. Typhi)* by detection of nucleic acid sequences related specifically to these serotypes genomic DNA. The invention further provides test kits for performing tests according to this method.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

METHOD OF TESTING FOR SALMONELLA.

The present invention relates to a method of testing for microorganisms of certain serotypes of the genus Salmonella by detection of their characteristic polynucleotide sequences. The invention further provides test kits for performing tests according to this method.

Salmonella typhi (S. typhi) is an invasive organism causing systemic disease, as opposed to the common salmonella intestinal disease, and is the major cause of enteric fever in humans. Oral ingestion of S. typhi organisms results in their invasion of the intestinal epithelium, entry into the circulatory system and colonization of the reticuloendothelial organs. After multiplication at these sites over a week or so they invade the blooodstream and cause septicemia, seeding the intestine through the gallbladder and bile duct, resulting in haemorrhage and perforation of the Peyer's patches in severe cases.

S. typhi organisms are usually recoverable from the blood and urine early in the disease and from the faecal stools between the third and fifth week of illness. It is found that about 2.5% of typhoid patients become chronic gallbladder carriers; those who are elderly or female being more susceptible to this condition. Significantly it is difficult to isolate S. typhi from such gallbladder carriers and thus, inter alia, provision of a rapid method for identification of these and patients in the early stages of the disease is desirable.

In the present applicant's copending applications PCT/GB 91/01690 (inventor Thorns) and PCT/GB 91/01691 (inventor Woodward) there are described methods for the detection of certain strains of salmonella by detection of a specific fimbrial antigen which occurs only in S. enteritidis, some strains of S. dublin and, as so far determined, only one strain each of S. moscow and S. blegdam; all other strains (several hundred) tested lacked this antigen. Both the latter serotypes which are very closely related to S. enteritidis, are extremely rare and have not been seen by the CVL's reference laboratory since the Zoonoses Order (1975) started in the United

Kingdom in 1976. The detection of strains expressing SEFA is therefore an indication of S. enteritidis or S. dublin, and indicates that a strain is not of S. typhi.

The present inventors have now surprisingly found that serotypes of S. typhi also have DNA encoding for this specific fimbrial antigen and thus that methods of copending application PCT/GB 91/01691 can be adapted for the detection of such organisms in a rapid and reliable manner. The antigen encoded is designated hereinafter as SEFA, Salmonella enteritidis Fimbrial Antigen, but the DNA coding for it is common to members of the enteritidis, dublin and typhi serogroups. Characterising data regarding SEFA is provided below, its amino acid sequence being given as SEQ. ID No 2, and the DNA sequence of the double stranded polynucleotide region containing the sequence encoding for it provided as SEQ. ID No 1 and SEQ. ID No 3 in the sequence listing attached hereto. The bases corresponding to the amino acid sequence are bases 73 to 600 of the SEQ ID No 1, corresponding to bases 1788 to 2315 of SEQ ID No 3.

The inventors have further now determined that although the SEFA antigen can be expressed in vitro by S. enteritidis, many S. dublin, and the isolated examples of S. moscow and S. blegdam, expression by S. typhi has so far been impossible to demonstrate, even when incubated in preferred expression supporting media of PCT/GB 91/01690.

Thus the present invention provides a method for testing for the presence of microorganisms of Salmonella serotype S. typhi comprising testing a sample for the presence of a nucleic acid sequence characteristic of genomic DNA from the region encoding for SEFA (SEQ ID No 1 and SEQ. ID No 3) and relating the presence of this to the presence of said serotype. Preferably, the relating of the presence of the characteristic sequence to presence of S. typhi will take into account the nature of the sample, and the likelihood of other SEFA encoding serotypes being present. Provision of further distinguishing steps provides a test for S. eneteritidis, S. typhi or S. dublin.

A particular method for determining the presence of polynucleotide sequences characteristic of genomic DNA from the region encoding for SEFA is provided by copending PCT/GB 91/01691 and comprises use of polynucleotide hybridization probes targeted to identify such sequences using techniques which are well known in the art. Examples of such probes are also exemplified below.

A further such method for determining the presence of such characteristic polynucleotide sequences comprises subjecting a sample of analyte to conditions under which polynucleotide sequences are replicated by use of specific sequence amplification reaction, for example the ligase or polymerase chain reactions (LCR and PCR), as provided by copending PCT/GB 91/01691. Such PCR amplication is well known in the art and suitable primers for use in PCR targeted at the sequences characteristic of SEFA are exemplified herein below.

The production of product in the presence of specific primers can be taken as a positive indication of the presence of the target sequence but it is posible to cross check this result by determination of further specific hybridization using probe sequences, such technique being well known in the art, using suitably different sequences selected from those exemplified for primer purposes. Methods for carrying out the PCR are conventional and illustrated by those described in EP-A-0201184 and EP-A-0200362 (both Cetus Corp).

Specific conditions for carrying out PCR on cells or DNA/RNA as provided in aqueous analyte samples are exemplified in EP 0409159 (Shimadzu) and EP 0438115 (Perkin Elmer), but variations of these will be known to a person skilled in the art of PCR based detection methods. The aqueous samples to which the present probes will be applied will typically be bodily fluids or food, faecal or tissue samples (eg. homogenates) but may potentially be any material from which cells or polynucleic acids may be derived.

As will be clear to those skilled in the art, it will not be necessary to employ probes and/or primers which are targeted at the whole of

this coding sequence in order to ascertain its presence. As is conventional in the art, probe and primer sequences can be targeted at any characeteristic sequence, prefably being of 12 or more bases long, more preferably 16 or more bases long, in so far as these are statistically unlikely to be found in interfering, non-targeted sequences. Thus preferred probes and/or primers consist of at least any contiguous 12, preferably any contiguous 16 bases of either of the respective strands of SEQ ID No 1 or SEQ ID No 3, preferably 73-600 of SEQ ID No 1 or its complement; primers obviously running 5'-3'.

It will be realised that an analyte determined to contain the SEFA characteristic sequences referred to above might equally comprise DNA/RNA or microorganisms of the other serotypes, most likely S. enteritidis or S. dublin. However, in practice, when analytes are provided they will be derived from or related to subjects showing symptoms which will be consistent with either S. typhi or S. enteritidis/S. dublin, but not both and thus a test may be carried out with a degree of certainty that a positive result will be indicative of the salmonella consistent with the particular symptoms. However, in the event that there are no symptoms to assist analysis (eg. where no live subject is available or where the samples are not directly derived from a human or animal body) or where it is desired to be more unequivocal in determination of the organisms present, the present invention further provides a method for determining the presence of an organism having DNA or RNA characteristic of that region encoding for SEFA and also determining its identity as being S. typhi, S.enteritidis or S. dublin comprising

(a) testing a sample of material under investigation for the presence of a nucleic acid sequence characteristic of the genomic DNA from the region encoding for SEFA (as SEQ. ID No 1 or SEQ ID No 3);

(b) testing that or a further sample of the material for the presence of an antigenic amino acid sequence or antibody thereto or a genomic nucleic acid sequence; said amino acid sequence or nucleic

acid sequence being associated with one or more of the S. enteritidis, S. dublin or S. typhi serotypes, but not found in all three;

and relating the results of tests (a) and (b) to the presence or absence of each of the three serotypes.

Thus step (a) may be carried out using any sequence determined to be characteristic of the genomic DNA of the region encoding for SEFA (as provided in SEQ ID 1 or SEQ ID No 3 below); these preferably being a sequence of suficient length to provide statistical probability of correct identification, ie. conventionally any sequence of 12 or more contiguous bases, more preferably of 16 or more bases, selected from this sequence.

Preferably the step (a) is carried out by use of specific sequence amplification, preferably PCR, and most preferably by carried out by use of PCR primers described below (SEQ ID 4, 5, 6, 7, 8, 9 and 10). Similarly these primers may be effectively employed as hybridization probe sequences when used in labelled form. Step (b) conveniently may be carried out using any distinguishing test utilising the differences in amino acid sequences characteristic of the particular serotypes.

As disclosed in PCT/GB 91/01690, S. enteritidis and S. dublin may be conveniently distinguished antigenically using a polyclonal antibody raised to S. dublin which is then absorbed with S. enteritidis in order to remove any antibody which crossreacts with the two. In this way a polyclonal antibody reactive with S. dublin is left that is not reactive with S. enteritidis. Similarly such polyclonal antibodies which are specific, as between the three 'common' SEFA encoding serotypes enteritidis, dublin and typhi, may be prepared by cross absorbing non-specific antibodies onto the two serotypes to which the antibodies were not raised in order to leave the desired characterising antibodies. Cross-absorption with the other two isolated strains of S. moscow and/or S. blegdam will also be advantageous, as will provision of specific antibodies for these.

More preferably monoclonal antibodies will be prepared using standard techniques to provide producing hybridoma cell lines such that antibodies all with the same target sequence and affinity may be used for the determinative tests. Of particular use are antibodies raised to the SEFA antigen or an epitope of that, in so far as these will bind to SEFA as expressed by all the strains of interest, except S. typhi, thus providing a ready check as to identity of the organism. These antibodies are subject of PCT/GB 91/01960 as described previously and hybridoma cells expressing one of these has been deposited in accordance with the Budapest Treaty at the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, WILTS SP4 0JQ, under accession number 90101101 on 11th October 1990.

In all cases of use of antibodies as reagents in these tests it will be possible to enhance visualisation of the antibody-antigen binding phenomena by labelling them with coloured latex particles as is known in the art (Hechemy K E and Michaelson (1984) Lab Management 22 27-40). Alternativley use may be made of secondary antibodies as is also known in the art whereby the secondary antibody is targeted at the antibody reagent and is labelled, eg. with gold, such that when excess unbound antibody is washed away, eg. so as to be removed from cells or fimbria in the sample, it is possible to observe the gold or other label clustered around the cells or fimbria thus indicating antibody binding on these.

As further explained in PCT/GB 91/01690, it is found that the content of the culture medium is a crucial factor in the in vitro production of SEFA epitopic sites on the Salmonella fimbria of the SEFA expressing strains. Peptone water and Enriched E broth (see Francis et al (1982) J. Clinical. Microbiol.. 15: 181-183) are examples of liquid media which will support relatively weak expression in reliable manner. Solid media examples include desoxycholate citrate agar, McConkey agar, Nutrient agar, Salmonella Shigella agar, Sheep blood agar, Xylose Lysine descholate. For more reliable and/or sensitive testing it may be necessary to use a medium that is more potent in supporting the expression; examples of such media being Oxoid

Isosensitest and Sensitest agars. Suitable media may be selected for the ability to support SEFA expression by S. enteritidis as determined by antigen-antibody binding assay using one of the monoclonal antibodies, eg. that deposited as referred to above or other SEFA derived specific antibodies.

The present invention further provides kits for performing the methods of this invention; kits for the determination of the presence of SEFA expressing organisms or their SEFA associated components (ie. SEFA or an epitopic part thereof, antibodies to SEFA or epitopic parts thereof, polynucleotides encoding for SEFA or epitopic parts thereof) being already provided by the aforesaid copending patent applications.

Thus kits of the present invention are characterised in that in addition to reagents for the determination of the presence of these SEFA encoding region characteristic sequences they, further comprise reagents for the determination of the presence of components distinctive of one or two of the three SEFA serotypes S. enteritidis, S.dublin and S. typhi such that the presence of each of these can be ascertained. Kits further containing recognition reagents for the S. moscow and/or S. blegdam are also provided for use on samples where the presence of these is a possibility.

The reagents for the determination of the presence of the SEFA encoding region characteristic polynucleotide sequences will comprise at least one or both of the following:

(i) specific sequence amplification reaction primers targeted at a nucleic acid sequence characteristic of the genomic DNA region encoding for SEFA (ie. characteristic of SEQ ID No 1 or SEQ ID No 3), and capable of intiating specific sequence amplification reaction production of these sequences in the presence of enzymes, such as polymerases or ligases;

(ii) hybridization probes targeted at characteristic polynucleotide sequences of the region encoding for SEFA (ie. of SEQ ID No 1 or SEQ

ID No 3) and capable of selectively hybridizing with these under test conditions.

Preferred kits comprising reagents for distinguishing a strain as being S. typhi may comprise antibodies capable of identifying one or more, but not all, of the SEFA encoding strains, or hybridoma cells capable of producing these. For example, the deposited cells referred to above or their antibodies which are optionally in labelled form (as is understood in the art), more preferably immobilised on solid carriers. Alternatively the kits contain labelling agents such as latex particles which may be coloured, for conveniently use.

The SEFA expression supporting media of PCT/GB 91/01960 may also be included for identifying the expressing strains, and thus providing evidence that S.typhi is not the strain present. Examples of preferred media capable of supporting SEFA expression are sold by Oxoid under the trade name 'Oxoid Sensitest Agar' and 'Oxoid Iso-Sensitest Agar'. The use of these media is described in PCT/GB 91/01960.

Culture of the Salmonella micro-organisms on the medium is by entirely standard conditions, eg by incubation at about 37°C until a sufficient number of the micro-organisms having epitopic sites on their fimbriae have grown, for example typically by overnight incubation. An incubation temperature of above 22°C is preferred for the effective production of the SEFA bearing fimbriae. In applying the test in practice, a sample of the analyte eg. a suspected bodily material would be taken, containing a cross-section of all the micro-organisms present in the material, and these would then be cultured on the medium so that the salmonella, if present, grows among any other micro-organisms that might be present. The presence of other micro-organisms does not seem to adversely affect the test.

Procedures for raising both polyclonal and monoclonal antibodies to salmonella surface antigens are well known. Thus, for example, S. enteritidis may be grown on a medium as described above so that

antigenic fimbriae are produced, these then may be used to immunise mice from which spleen cells are subsequently isolated and fused with a myeloma cell line to form hybridomas. These hybridomas may then be seeded into microwells and monitored for antibody production, eg by ELISA or a similar technique. Antibody-producing hybridomas may then be cloned to produce a mouse monoclonal antibody to the Salmonella fimbrial antigen. MABs may be produced by the known method of intraperitoneally injecting hybridoma 10 cells (eg; 10^6) into mice and withdrawing ascites after 20 days; this can be used in crude form if necessary.

The exemplified monoclonal antibodies use further extends to (i) the determination of media suitable for growing salmonella possessing the required antigenic fimbriae and (ii) for identification of said antigenic fimbriae and antigens comprising the SEFA epitope itself. Thus further specific media suitable for the performance of the method of the invention may be easily identified by screening salmonella grown in them for the ability to produce immunoagglutination with said MABs; a positive result indicating a suitable medium. Either the whole Salmonella micro-organisms (live or dead) or a part thereof which includes the fimbrial antigen with the SEFA epitopic site may be detected by the antibody. In the latter case methods are well known, eg. mild heat shock treatment at 60°C for 30 minutes, for detaching fimbriae from salmonella micro-organisms, and isolation of the fimbrial antigen in this way should lead to a more specific test result.

The epitopic sites employed in this method are present on a fimbrial structure produced on the surface of S. enteritidis grown on the media described above and in vivo, which is less than 6 nm in diameter and consists of identical repeating subunits each of molecular weight between 14,000 and 15,000. These fimbriae have a 'kinked' conformation such that they entangle and extend in a matted form to approximately 200nm from the cell surface. By applying size exclusion HPLC and SDS-PAGE to the fimbrial antigen isolated in such a way it has been determined that the SEFA antigenic protein employed

appears to have a molecular weight of approximately 14,300. Isolated SEFA as described here has a major antigenic activity and its amino acid sequence is given below.

In use of SEFA or an epitopic part thereof to determine the presence of SEFA antibodies in an analyte sample, the antigen in the form of whole micro-organisms, the isolated fimbriae or isolated SEFA or epitopic part thereof may be immobilised on a substrate such as a microtitre plate well, using known methods. This immobilised antigen may be exposed to a solution suspected of containing the SEFA antibody, then after washing a second labelled antibody capable of binding to the unlabelled SEFA antibody may be applied (eg: a labelled anti-human Ig G) to the wells. After further washing detection of binding between this second antibody and the SEFA antibody itself bound to the immobilised antigen may then be observed by the presence of the bound label on the well. Other antibody/second antibody combinations will occur to the man skilled in the art (eg murine bovine or chicken antibodies/anti-murine anti-bovine or anti-chicken second antibodies).

In a yet further way the antibody may be immobilised on a substrate and the immobilised antibody may then be exposed to a solution containing the antigen in the form of for example whole micro-organisms, the isolated fimbriae or the antigenic protein (SEFA), together with an agent capable of competing with the antigen for binding sites on the antibody. The quantity of the agent binding to the immobilised antibody may then be determined, eg: by use of known, labelling techniques. For example the competing agent may be a labelled anti-mouse IgG if the antibody is a mouse monoclonal, or may be labelled fimbrial antigen. The labels used in the above methods may be entirely conventional, and ways of labelling antibodies are well known.

The test kits may contain further reagents and other items for performance of the two or more determinations necessary (ie. the SEFA determination and the serotype enteritidis /dublin/typhi

determination). For example as well the antibodies and the SEFA expression medium, visualising agents and standard result cards may be included. Depending upon the way in which the test is to be applied the antibody may be provided in the form of a solution, eg, for immunoagglutination or if the antigen is to be immobilised, or the antibody may be provided in the aforementioned immobilised form. The test kit may optionally also contain a further antibodies for further cross-checking salmonella serotype, instructions and appropriate vessels for carrying out the test.

In the method of the present invention the presence of nucleic acid sequences characteristic of the genomic DNA from the region encoding SEFA, as defined by SEQ ID No 2 in the listing below, and preferably of the 527 basepair DNA sequence actually encoding for SEFA, as illustrated in SEQ ID No 1 , is used to determine the presence of one of the aforesaid salmonella serotypes, and the serotype is further differentiated to determine the presence of S. typhi or one of the expressing strains.

The presence of one of the SEFA encoding region serotypes can be ascertained by use oligonucleotide probes and primers for the purpose of detecting SEFA encoding serotype genomic DNA. As well as the key characteristic sequences so defined sequences encoding for allelic variants of SEFA will also be characteristic of such serotypes.

The polynucleotide sequence directly corresponding to SEFA is on the upper strand as shown above beginning ATGCTAATAG and ending GTATCAAAAC in SEQ ID No 1. PCT/GB 91/01691 provides recombinant DNA, plasmids and methods using them for genetically engineering organisms capable of expressing SEFA.

That patent further provides methods for determining the presence of microorganisms having DNA or RNA polynucleotide sequence encoding for SEFA or an epitopic part thereof, allelic variants of these, or such DNA or RNA itself, comprising: (a) providing a sample suspected of containing said encoding polynucleotide sequence; (b) determining the

presence of said sequence by monitoring hybridization of SEFA targeted polynucleotide probes to it.

Such hybridization technique is carried out by methods that are now conventional in the art, using probes which are comprised of sequences complementary to a significant part of the target sequence and using temperature conditions suitable to achieved a desired stringency dependent on the degree of match of the probe to the target. Probes complementary to the length up to the entire target sequence may be used.

Conveniently the characteristic sequence is detected, in both amplified and unamplified tests, by use of a hybridization probe suitably specific thereto which comprises any of the aforementioned sequences, more specifically being one of the sequences in a suitably labelled form eg. being labelled in some way as will be known to a man skilled in the art. Most conveniently the label will incorporate radioactive phosphorous (^{32}P). A preferred such method comprises a PCR step (b) which employs primer pairs targeted to amplify characteristic sequences, a particularly preferred method comprises use of one primer selected from groups (A) and the other from group(B):

Group A(SEQ ID No):	Group B(SEQ ID No):
A4: 5' -GTGCGAATGCTAATAGTTGA- 3'	B8: 5' -AAAACAGGCTGTCCTTGTCCA- 3'
A5: 5' -TGCGTAAATCAGCATCTGCA- 3'	B9: 5' -TTAGCGTTTCTTGAGAGCTGG- 3'
A6: 5' -TCTGCAGTAGCAGTTCTTGC- 3'	B10:5' -TTTTGATACTGCTGAACGTAG- 3'
A7: 5' -GCTCAGAATACAACATCAGCCAA- 3'	

These are designated SEQ ID No 4 to 7 (A4-A7) and SEQ ID No 8 to 10 (B8-B10) respectively in the listing below.

Any of the possible pairs selected in this way will bind with the characteristic sequences sufficiently specifically enough for serotype determination purposes using PCR under standard conditions, ie: for determination of a salmonella as being a SEFA encoding serotype and thus of one of the serotypes listed above.

As will be understood by a man skilled in the art, sequences which will specifically hybridize with the characteristic sequences will include those sequences themselves, those having high eg.75% or more, preferably 90% or more conformity to such sequence, and sequences comprising either strand of the two complementary sequences of any of these. Thus the step (c) of the method of this aspect of the invention may be carried out using a variety of hybridization probes that combine sufficiently specifically with the characteristic 'target' sequence. For most purposes the primer sequences selected from those of groups (A) and (B) will be sufficiently specific to give reliable determination of the characteristic sequence, especially if a different 'primer' sequence is used for the probe of step (c) than those used for step (b).

In this way substantially larger quantities of the DNA sequence may be made from the small quantities which may be available by isolation from the S. enteritidis , S. dublin or S.typhi thus increasing the amount of sequence available to be detected. The mere presence of increased amount of DNA may be used in this case to signify presence of target sequence.

Comparison of S. typhi and S. enteritidis DNA. In order to demonstrate the similarity between the SEFA encoding regions of genomic DNA from S. enteritidis and S. typhi, the following experimental was carried out.

1. Genomic DNA was extracted from S. typhi Ty21a using a gentle lysis protocol described by Goldberg and Ohman (1984 J. Bact 158 1115-1121) and resuspended in TE (10mM Tris pH 8, mM EDTA) to a final concentration of 2μg μl^{-1}.

2. Samples of S. typhi DNA (5μg) were denatured by adjusting to pH 2.5 by addition of 1M NaOH to give a final concentration of 0.3M. the DNA sample was spotted onto nylon filters, allowed to air dry and the DNA fixed by UV irradiation.

3. samples of positive control DNA from S.enteritidis and negative control DNA from E. coli were treated as in 2. above and spotted onto the same filter in distinct locations. 4. A 583 basepair DNA fragment encoding the entire SEFA fimbrial antigen removed as a DraI excision product was radiolabelled using ^{32}P by the random hexanucleotide labelling system (Amersham) as described by Feinberg and Vogelstein (1983, Analytical Biochem. 132, 6-13). The labelled DNA was used in DNA:DNA hybridization experiments using the filter prepared in 2. and 3. above following standard methods Woodward and Sullivan, 1991 J. Gen. Micro. 137. 1101-1109). Post hybridization washes were at 65°C with 0.1 x 3SO/0.1% SDS as the final wash solution. 5. Washed filters were air dried and then exposed to X-ray film (Fuji-RX. RTM). Exposed film was developed using standard photographic procedures. DNA from S.enteritidis and S. typhi bound the probe with equal activity while the negative control DNA did not bind it at all.

6. Total genomic DNA (5µg) extracted from S.typhi Ty21a was digested to completion with various restriction endonucleases in separate experiments. Digested DNA was fractionated by standard gel electrophoresis, Southern blotted and hybridized as above (4. and 5.) Unique DNA bands bound the probe.

7. S.typhi genomic DNA (100ng) was used in PCR experiments with the oligonucleotide primers of groups A and B as herein described. In each experiment, using standard conditions (Taiki et al. 1985. Science 230: 1350-1354) amplified products of the desired size were obtained and each product was shown by Southern hybridization to be homologous with the 583 base pair probe.

Thus experiments 1. to 7. demonstrate the common SEFA encoding sequence as being present in S.typhi as well as the previously determined presence in S. enteritidis and S. dublin.

Thus when seeking to differentiate the three SEFA serotypes by use of a polynucleotide sequence encoding for a specific antigenic amino-acid

sequence associated with one or two of them, but not all three it is clear that SEFA is likely to be of no utility. Conveniently sequences will thus be those for the p and G antigens referred to above in the immunological tests.

The various aspects of the invention will now be described by way of the following non-limiting protocol examples.

EXAMPLE 1: Test kit reagents and protocol for use:

A kit for determination of presence of S. typhi is provided as follows. Probes/primers are selected from groups (A) and (B) above for use with standard laboratory reagents for hybridization probing and/or PCR reaction. Such probes may be purchased to order from companies such as Pharmacia UK, or synthesized according to standard techniques (see Gait M J (Edit.) 'Oligonucleotide synthesis- a practical approach', IRL Press, Oxford (1984) and Beaucage & Carruthers, Tetrahedron Letters 22 p1859-1862). Radiolabelling and Southern blotting are carried out by conventional methods.

Kit reagents provided for the purpose of identifying the SEFA encoding strain as of serotype S. typhi, or as one of the SEFA expressing strains, comprise monoclonal antibody directed at SEFA, a polyclonal antibody directed to S. dublin flagella p antigen but not immunoreactive with enteritidis or typhi, a polyclonal antibody directed at G component of S. dublin and S. enteritidis flagella but not immunoreactive with S. typhi, reader cards and preferred growth medium optionally with any of the reagents (eg latex particles) below used in the test.

Coating of latex: To prepare a batch of latex coated with any of the antibodies. Materials: Glycine bufferred saline (GBS as above), Bovine serum albumen (fatty acids free) (Code A-6003, Sigma Chemicals), coloured latex (colour chosen to identify a particular antibody on its surface), 0.8microns, 10% suspension (Code K080, Estapor, Rhone-Poulene), antibody containing fluid, Glass container of

the suitable size - Pressmatic dispenser (Bibby) - Dropper bottles - Labels - Rocking device

Method: volumes of latex, antibody and GBS appropriate for that batch size are mixed in a glass container and incubated for 2 hours at 37°C with constant gentle rocking, centrifuged for 20 minutes at 3500 rpm. The supernatant is discarded and the latex resuspended in appropriate volume of GBS containing 0.1% BSA ready for use. Control latex may be prepared by coating with normal mouse serum collected from 8-12 week old Balb/c mice.

Positive control SEFA, p protein (re dublin) or G component (re enteritidis and dublin) is/are preferably included in the kit or a sample of a salmonella capable of producing them may be provided with a suitable media for enabling SEFA expression for control test purposes.

EXAMPLE 2: Use of test kit of Example 1.

Samples testing positive, as including SEFA coding region characteristic sequences in tests using probes or PCR amplification, were designated as containing organisms of the S. enteritidis/S. dublin expressing type or of S. typhi, and thus put forward for differentiation by the following.

Samples were exposed to test latexes as prepared above are compared with controls using reader cards. One test latex is used to identify presence of SEFA bearing materials (eg; whole organisms). The further test latexes are used to differentiate S. enteritidis, S. dublin and S. typhi. In this protocol S. typhi does not react with any of the antibodies, although other protocols using positive identification will occur to those skilled in the art. The control latex aids determination of false positives caused by, inter alia, autoagglutination. The positive control and reader cards are used to determine degree of response.

Note; other commercially available antisera are available which are capable of differential binding with these three significant SEFA expressing serotypes; for example: Northumbria Biologicals Ltd UK -Pasteur Products- supply:

Product number	Antisera	Distinguishes
2061121	Monovalent H antisera g.m	enter' from dublin
2061117	Monovalent H antisera m	enter' from dublin+typhi
2061118	Monovalent H antisera p	dublin from enter'+typhi

Further such antisera are available from Wellcome Reagents Ltd and are coded in their AL/ZD codes: AL47,48,49/ZD13,14,15.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: THE MINISTER OF AGRICULTURE FISHERIES & FOOD IN HER BRITANN
(B) STREET: WHITEHALL PLACE
(C) CITY: LONDON
(E) COUNTRY: GB
(F) POSTAL CODE (ZIP): SW1A 2HH

(A) NAME: MARTIN JOHN WOODWARD
(B) STREET: 23 BURNSALL CLOSE
(C) CITY: FARNBOROUGH
(D) STATE: HAMPSHIRE
(E) COUNTRY: GB
(F) POSTAL CODE (ZIP): GU14 8NN

(A) NAME: CHRISTOPHER JOHN THORNS
(B) STREET: 11 LINCOLN DRIVE
(C) CITY: PYRFORD, WOKING
(D) STATE: SURREY
(F) POSTAL CODE (ZIP): GU22 8RL

(ii) TITLE OF INVENTION: METHOD OF TESTING FOR SALMONELLA (iii)

NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS (D) SOFTWARE: PatentIn Release £1.0, Version £1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9207069.7 (B) FILING DATE: 31-MAR-1992

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2387 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 73..600

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 1: FROM 1 TO 2387

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 1: FROM 1 TO 2387

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GATCCTTGTT TTTTTTCTTA AATTTTTAAA ATGGCGTGAG TATATTAGCA TCCGCACAGA      60

TAAATTGTGC GA ATG CTA ATA GTT GAT TTT TGG AGA TTT TGT AAT ATG           108
              Met Leu Ile Val Asp Phe Trp Arg Phe Cys Asn Met
                1               5                  10

CGT AAA TCA GCA TCT GCA GTA GCA GTT CTT GCT TTA ATT GCA TGT GGC         156
Arg Lys Ser Ala Ser Ala Val Ala Val Leu Ala Leu Ile Ala Cys Gly
        15                  20                  25

AGT GCC CAC GCA GCT GGC TTT GTT GGT AAC AAA GCA GAG GTT CAG GCA         204
Ser Ala His Ala Ala Gly Phe Val Gly Asn Lys Ala Glu Val Gln Ala
    30                  35                  40

GCG GTT ACT ATT GCA GCT CAG AAT ACA ACA TCA GCC AAC TGG AGT CAG         252
Ala Val Thr Ile Ala Ala Gln Asn Thr Thr Ser Ala Asn Trp Ser Gln
45                  50                  55                  60

GAT CCT GGC TTT ACA GGG CCT GCT GTT GCT GCT GGT CAG AAA GTT GGT         300
Asp Pro Gly Phe Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val Gly
                65                  70                  75

ACT CTC AGC ATT ACT GCT ACT GGT CCA CAT AAC TCA GTA TCT ATT GCA         348
Thr Leu Ser Ile Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile Ala
            80                  85                  90

GGT AAA GGG GCT TCG GTA TCT GGT GGT GTA GCC ACT GTC CCG TTC GTT         396
Gly Lys Gly Ala Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val
        95                  100                 105

GAT GGA CAA GGA CAG CCT GTT TTC CGT GGG CGT ATT CAG GGA GCC AAT         444
Asp Gly Gln Gly Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn
    110                 115                 120

ATT AAT GAC CAA GCA AAT ACT GGA ATT GAC GGG CTT GCA GGT TGG CGA         492
Ile Asn Asp Gln Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg
125                 130                 135                 140

GTT GCC AGC TCT CAA GAA ACG CTA AAT GTC CCT GTC ACA ACC TTT GGT         540
Val Ala Ser Ser Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe Gly
                145                 150                 155

AAA TCG ACC CTG CCA GCA GGT ACT TTC ACT GCG ACC TTC TAC GTT CAG         588
```

Lys Ser Thr Leu Pro Ala Gly Thr Phe Thr Ala Thr Phe Tyr Val Gln
160 165 170

CAG TAT CAA AAC TAATTTAATT TAAACTTTAT AAATGCCCTC AATATGAGCG 640
Gln Tyr Gln Asn
175

AGTTTGGATA ATTTTATTAT TTTAAAAATA TCTATTTTGA ATAGATAGGT TTTATGCTTC 700

CATGCAAAAA CTTAAAGAGG GATTATGTAT ATTTTGAATA AATTTATACG TAGAACTGTT 760

ATCTTTTTCC TTTTTTTTGC TACCTTCCAA TTGCTTCTTC GGAAAGTAAA AAAATTGAGC 820

AACCATTATT AACACAAAAA TATTATGGCC TAAGATTGGG CACTACACGT GTTATTTATA 880

AAGAAGATGC TCCATCAACA AGTTTTTGGA TTATGAATGA AAAAGAATAT CCAATCCTTG 940

TTCAAACTCA AGTATATAAT GATGATAAAT CATCAAAAGC TCCATTTATT GTAACACCAC 1000

CTATTTTGAA AGTTGAAAGT AATGCGCGAA CAAGATTGAA GGTAATACCA ACAAGTAATC 1060

TATTCAATAA AAATGAGGAG TCTTTGTATT GGTTGTGTGT AAAAGGAGTC CCACCACTAA 1120

ATGATAATGA AAGCAATAAT AAAAACAACA TAACTACGAA TCTTAATGTG AATGTGGTTA 1180

CGAATAGTTG TATTAAATTA ATTTATAGGC CTAAAACTAT AGACTTAACG ACAATGGAGA 1240

TTGCAGATAA ATTAAAGTTA GAGAGAAAAG GAAATAGTAT AGTTATAAAG AATCCAACAT 1300

CATCATATGT GAATATTGCA AATATTAAAT CTGGTAATTT AAGTTTTAAT ATTCCAAATG 1360

GATATATTGA GCCATTTGGA TATGCTCAAT TACCTGGTGG AGTACATAGT AAAATAACTT 1420

TGACTATTTT GGATGATAAC GGCGCTGAAA TTATAAGAGA ATTATTAGTT TAAGGTGTAA 1480

AACAAATGAA GAAAACCACA ATTACTCTAT TTGTTTTAAC CAGTGTATTT CACTCTGGAA 1540

ATGTTTTCTC CAGACAATAT AATTTCGACT ATGGAAGTTT GAGTCTTCTC CCGGTGAGAA 1600

TGCATCTTTT CTAAGTGTTG AAACGCTTCC CTGGTAATTA TGTTGTTGAT GTATATTTGA 1660

ATAATCAGTT AAAAGAAACT ACTGAGTTGT ATTTCAAATC AATGACTCAG ACTCTAGAAC 1720

CATGCTTAAC AAAAGAAAAA CTTATAAAGT ATGGGATCGC CATCCAGGAG CTTCATGGGT 1780

TGCAGTTTGA TAATGAACAA TGCGTTCTCT TAGAGCATTC TCCTCTTTAA ATATACTTAT 1840

AACGCGGCTA ACCAAAGTTT GCTTTTAAAT GCACCATCTA AAATTCTATC TCCAATAGAC 1900

AGTGAAATTG CTGATGAAAA TATCTGGGAT GATGGCATTA ACGCTTTTCT TTTAAATTAC 1960

AGAGCTTAAT TATTTGCATT CTAAGGTTGG AGGAGAGAGA TTCATACTTT GGTCAAATTC 2020

AACCTTGGTT TTAATTTTGG TCCCTGGCGG CTAAGGAATC TATCATCTTG GCAAAACTTG 2080

TCAAGCGAAA AAAAATTTGA ATCAGCATAT ATTTATGCTG AGCGAGGTTT AAAAAAAATA 2140

AAGAGCAAAC TAACAGTTGG GGACAAATAT ACCAGTGCAG ATTTATTCGA TAGCGTACCA 2200

TTTAGAGGCT TTTCTTTAAA TAAAGATGAA AGTATGATAC CTTTCTCACA GAGAACATAT 2260

TATCCAACAA TACGTGGTAT TGCGAAAACC AATGCGACTG TAGAAGTAAG ACAAAATGGA 2320

TACTTGATAT ATTCTACTTC AGTCCCCCCC GGGCAATTCG AGATAGGTAG AGAACAAATT 2380

CTGATC 2387

2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Leu Ile Val Asp Phe Trp Arg Phe Cys Asn Met Arg Lys Ser Ala
1 5 10 15

Ser Ala Val Ala Val Leu Ala Leu Ile Ala Cys Gly Ser Ala His Ala
20 25 30

Ala Gly Phe Val Gly Asn Lys Ala Glu Val Gln Ala Ala Val Thr Ile
35 40 45

Ala Ala Gln Asn Thr Thr Ser Ala Asn Trp Ser Gln Asp Pro Gly Phe
50 55 60

Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val Gly Thr Leu Ser Ile
65 70 75 80

Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile Ala Gly Lys Gly Ala
85 90 95

Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val Asp Gly Gln Gly
100 105 110

Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn Ile Asn Asp Gln
115 120 125

Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg Val Ala Ser Ser
130 135 140

Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe Gly Lys Ser Thr Leu
145 150 155 160

Pro Ala Gly Thr Phe Thr Ala Thr Phe Tyr Val Gln Gln Tyr Gln Asn
165 170 175

2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2387 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

i) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 3: FROM 1 TO 2387

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 3: FROM 1 TO 2387

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GATCAGCAAT TTGTTCTCTA CCTATCTCGA ATTGCCCGGG GGGGACTGAA GTAGAATATA 60

TCAAGTATCC ATTTTGTCTT ACTTCTACAG TCGCATTGGT TTTCGCAATA CCACGTATTG 120

TTGGATAATA TGTTCTCTGT GAGAAAGGTA TCATACTTTC ATCTTTATTT AAAGAAAAGC 180

CTCTAAATGG TACGCTATCG AATAAATCTG CACTGGTATA TTTGTCCCCA ACTGTTAGTT 240

TGCTCTTTAT TTTTTTTAAA CCTCGCTCAG CATAAATATA TGCTGATTCA AATTTTTTTT 300

CGCTTGACAA GTTTTGACAA GATGATAGAT TCCTTAGCCG CCAGGGACCA AAATTAAAAC 360

CAAGGTTGAA TTTGACCAAA GTATGAATCT CTCTCCTCCA ACCTTAGAAT GCAAATAATT 420

AAGCTCTGTA ATTTAAAAGA AAAGCGTTAA TGCCATCATC CCAGATATTT TCATCAGCAA 480

TTTCACTGTC TATTGGAGAT AGAATTTTAG ATGGTGCATT TAAAAGCAAA CTTTGGTTAG 540

CCGCGTTATA AGTATATTTA AAGAGGAGAA TGCTCTAAGA GAACGCATTG TTCATTATCA 600

AACTGCAACC CATGAAGCTC CTGGATGGCG ATCCCATACT TTATAAGTTT TTCTTTTGTT 660

AAGCATGGTT CTAGAGTCTG AGTCATTGAT TTGAAATACA ACTCAGTAGT TTCTTTTAAC 720

TGATTATTCA AATATACATC AACAACATAA TTACCAGGGA AGCGTTTCAA CACTTAGAAA 780

AGATGCATTC TCACCGGGAG AAGACTCAAA CTTCCATAGT CGAAATTATA TTGTCTGGAG 840

AAAACATTTC CAGAGTGAAA TACACTGGTT AAAACAAATA GAGTAATTGT GGTTTTCTTC 900

ATTTGTTTTA CACCTTAAAC TAATAATTCT CTTATAATTT CAGCGCCGTT ATCATCCAAA 960

ATAGTCAAAG TTATTTTACT ATGTACTCCA CCAGGTAATT GAGCATATCC AAATGGCTCA 1020

ATATATCCAT TTGGAATATT AAAACTTAAA TTACCAGATT TAATATTTGC AATATTCACA 1080

TATGATGATG TTGGATTCTT TATAACTATA CTATTTCCTT TTCTCTCTAA CTTTAATTTA 1140

TCTGCAATCT CCATTGTCGT TAAGTCTATA GTTTTAGGCC TATAAATTAA TTTAATACAA 1200

CTATTCGTAA CCACATTCAC ATTAAGATTC GTAGTTATGT TGTTTTTATT ATTGCTTTCA 1260

TTATCATTTA GTGGTGGGAC TCCTTTTACA CACAACCAAT ACAAAGACTC CTCATTTTTA 1320

TTGAATAGAT TACTTGTTGG TATTACCTTC AATCTTGTTC GCGCATTACT TTCAACTTTC 1380

AAAATAGGTG GTGTTACAAT AAATGGAGCT TTTGATGATT TATCATCATT ATATACTTGA 1440

GTTTGAACAA GGATTGGATA TTCTTTTTCA TTCATAATCC AAAAACTTGT TGATGGAGCA 1500

TCTTCTTTAT AAATAACACG TGTAGTGCCC AATCTTAGGC CATAATATTT TTGTGTTAAT 1560

AATGGTTGCT CAATTTTTTT ACTTTCCGAA GAAGCAATTG GAAGGTAGCA AAAAAAAGGA 1620

AAAAGATAAC AGTTCTACGT ATAAATTTAT TCAAAATATA CATAATCCCT CTTTAAGTTT 1680

TTGCATGGAA GCATAAAACC TATCTATTCA AAATAGATAT TTTTAAAATA ATAAAATTAT 1740

CCAAACTCGC TCATATTGAG GGCATTTATA AAGTTTAAAT TAAATTAGTT TTGATACTGC 1800

TGAACGTAGA AGGTCGCAGT GAAAGTACCT GCTGGCAGGG TCGATTTACC AAAGGTTGTG 1860

ACAGGGACAT TTAGCGTTTC TTGAGAGCTG GCAACTCGCC AACCTGCAAG CCCGTCAATT 1920

CCAGTATTTG CTTGGTCATT AATATTGGCT CCCTGAATAC GCCCACGGAA AACAGGCTGT 1980

CCTTGTCCAT CAACGAACGG GACAGTGGCT ACACCACCAG ATACCGAAGC CCCTTTACCT 2040

GCAATAGATA CTGAGTTATG TGGACCAGTA GCAGTAATGC TGAGAGTACC AACTTTCTGA 2100

CCAGCAGCAA CAGCAGGCCC TGTAAAGCCA GGATCCTGAC TCCAGTTGGC TGATGTTGTA 2160

TTCTGAGCTG CAATAGTAAC CGCTGCCTGA ACCTCTGCTT TGTTACCAAC AAAGCCAGCT 2220

GCGTGGGCAC TGCCACATGC AATTAAAGCA AGAACTGCTA CTGCAGATGC TGATTTACGC 2280

ATATTACAAA ATCTCCAAAA ATCAACTATT AGCATTCGCA CAATTTATCT GTGCGGATGC 2340

TAATATACTC ACGCCATTTT AAAAATTTAA GAAAAAAAAC AAGGATC 2387

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 4: FROM 1 TO 20

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 4: FROM 1 TO 20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GTGCGAATGC TAATAGTTGA 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 5: FROM 1 TO 20

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 5: FROM 1 TO 20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TGCGTAAATC AGCATCTGCA 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 6: FROM 1 TO 20

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 6: FROM 1 TO 20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCTGCAGTAG CAGTTCTTGC 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 7: FROM 1 TO 23

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 7: FROM 1 TO 23

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCTCAGAATA CAACATCAGC CAA 23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 8: FROM 1 TO 21

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 8: FROM 1 TO 21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAAACAGGCT GTCCTTGTCC A 21

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 9: FROM 1 TO 2387

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A

(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 9: FROM 1 TO 2387

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTAGCGTTTC TTGAGAGCTG G 21

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Salmonella enteritidis/Salmonella typhi

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206197 A1
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 10: FROM 1 TO 2387

(x) PUBLICATION INFORMATION:
(H) DOCUMENT NUMBER: WO 9206198 A
(I) FILING DATE: 01-OCT-1991
(J) PUBLICATION DATE: 16-APR-1992
(K) RELEVANT RESIDUES IN SEQ ID NO: 10: FROM 1 TO 2387

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
TTTTGATACT GCTGAACGTA G 21

CLAIMS

1. A method for testing for the presence of microorganisms of salmonella serotype S. typhi comprising testing a sample of a material under investigation for the presence of a nucleic acid sequence characteristic of the genomic DNA from the region encoding for SEFA (amino acid SEQ ID No 2) or its alleles and relating the presence of any of these to the presence of that serotype.

2. A method as claimed in claim 1 wherein the sample is tested for the presence of a nucleic acid sequence characteristic of either one of SEQ ID No 1 and SEQ ID No 3.

3. A method as claimed in claim 1 wherein the sequences characteristic of the SEFA encoding region are present within the sequence that directly encodes for SEFA, as herein described in SEQ ID No 1 as bases 73 to 600 or in SEQ ID No 3 as bases 1788 to 2315.

4. A method as claimed in any one of claims 1 to 3 comprising exposing DNA or RNA derived from the material to one or more hybridization probes targeted at said sequence and relating the occurrence of specific hybridization to the presence of said serotype.

5. A method as claimed in any one of claims 1 to 3 comprising subjecting the sample to conditions under which the characteristic sequences are replicated by use of a specific sequence amplification reaction and relating the production of amplification product to the presence of S. typhi.

6. A method as claimed in claim 5 wherein the specific sequence amplification reaction is a polymerase chain reaction.

7. A method as claimed in claim 5 or claim 6 wherein the identity of any replicate sequence produced by the amplification reaction is determined by exposing it to polynucleotide hybridization probes targeted at it and relating the occurrence of specific hybridization to the presence of that sequence.

8. A method for testing for the presence of microorganisms and/or nucleic acids of salmonella serotypes S. typhi, S. enteritidis or S. dublin comprising:

(a) testing a sample of a material under investigation for the presence of a nucleic acid sequence characteristic of the genomic DNA from the region encoding for SEFA (amino acid SEQ ID No 2) or its alleles and relating the presence of any of these to the presence of one of these serotypes;

(b) testing the sample or a further sample of the material for the presence of an antigenic amino acid sequence or antibody thereto or a polynucleotide sequence encoding for such antigenic amino acid sequence, said amino acid sequence being associated with one or more of the S. enteritidis, S. dublin or S. typhi serotypes, but not found in all three, and relating the presence of this to the presence of S. typhi or S. enteritidis or S. dublin.

9. A method as claimed in claim 8 wherein step (a) is carried out by testing the sample for the presence of a nucleic acid sequence characteristic of either one of SEQ. ID No 1 or SEQ. ID No 3.

10. A method as claimed in claim 8 or 9 wherein the amino acid sequence comprises that of S. dublin p protein or S. dublin/S. enteritidis G component.

11. A method as claimed in any one of claims 8 to 10 wherein for step (b) a culture medium capable of supporting expression of SEFA by S. enteritidis or S. dublin is innoculated with a sample of the material under investigation and incubated, the resultant medium being tested for expressed SEFA.

12. A method as claimed in claim 11 wherein the culture medium consists of Peptone water and Enriched E broth, desoxycholate citrate agar, McConkey agar, Nutrient agar, Salmonella Shigella agar, Sheep blood agar, Xylose Lysine descholate, Oxoid Isosensitest agar or Oxoid Sensitest agar.

13. A method as claimed in claim 11 wherein the culture medium consists of Oxoid Isosensitest agar or Oxoid Sensitest agar.

14. A method as claimed in any one of claims 11, 12 or 13 wherein the culture is carried out at a temperature greater than 22°C.

15. A method as claimed in any one of claims 4 to 7 wherein the probes or primer sequences are selected from SEQ ID Nos 4 to 10.

16. A method as claimed in either of claims 6 and 7 wherein the primers are selected one from each of groups (A) and (B):

Group A:	Group B:
SEQ ID No 4	SEQ ID No 8
SEQ ID No 5	SEQ ID No 9
SEQ ID No 6	SEQ ID No 10
SEQ ID No 7	

17. A method as claimed in any one of claims 4 or 7 wherein the probe selected from sequences of either of groups A or B described in Claim 15 provided that where the polymerase chain reaction is used the probe sequence is different to that of either of the primers used for step (b).

18. A test kit for performing a test as claimed in any one of claims 1 to 17 comprising one or both of:

(a) polymerase chain reaction probes targeted at characteristic parts of polynucleotide sequences encoding for SEFA or an epitopic part thereof and capable of intiating polymerase chain reaction production of these sequences in the presence of taq polymerase and

(b) hybridization probes targeted at characteristic parts of polynucleotide sequences encoding for SEFA or an epitopic part thereof and

18. A test kit as claimed in claim 17 further comprising one or more of:

(i) antibodies to SEFA or an epitopic part thereof or cells capable of producing those antibodies;

(ii) SEFA or an epitopic part thereof in the form of cells, fimbria isolated SEFA or said part or any of these immobilised onto a surface;

(iii) secondary antibodies capable of specific binding to the antibodies to SEFA or to antibodies to the epitopic part thereof and

(vi) medium or media capable of supporting or switching off expression of SEFA by S. enteritidis and/or S. dublin or essential components for preparing such medium or media.

19. A test kit as claimed in claim 18 wherein the antibodies are immobilised on a solid carrier.

20. A test kit as claimed in claim 18 or 19 further comprising an antibody labelling agent.

21. A test kit as claimed in Claim 20 wherein the labelling agent comprises latex particles.

22. A test kit as claimed in any one of Claims 18 to 21 wherein the antibodies are in labelled form.

23. A test kit as claimed in Claim 22 wherein the components comprise the dry components for preparation of peptone water pH 7.2, peptone water pH 6.0 or a Medium B (as herein described).

24. A test kit as claimed in Claim 23 wherein the Medium B is Sensitest agar or Isosensitest agar.

25. A test kit as claimed in Claim 18 wherein the SEFA or epitopic part thereof or antibodies thereto are immobilised on a microtitre plate.

26. A test kit as claimed in claim 18 comprising hybridization probes targeted at sequences characteristic of SEQ. ID 2 or SEQ. ID No 3.

27. A test kit as claimed in claim 26 wherein the probes comprise sequences comprising sequence directly coding for SEFA.

28. A test kit as claimed in claim 18 comprising primers and probes having sequences selected from the groups (A) and (B) set out in claim 16.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00647

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68; G01N33/569

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category [o]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	WO,A,9 201 056 (INSTITUT PASTEUR) 23 January 1992 see page 5, line 6 - page 10, line 33; claims	1
A	EP,A,0 383 509 (ORTHO DIAGNOSTICS SYSTEMS) 22 August 1990 see page 2, line 45 - page 4, line 13; claims; table 2	1
P,A	WO,A,9 206 198 (THE MINISTER FOR AGRICULTURE OF GREAT BRITAIN AND NORTHERN IRELAND) 16 April 1992 cited in the application see the whole document	1

-/--

[o] Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 JULY 1993	06. 08. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,A	WO,A,9 206 197 (THE MINISTER OF AGRICULTURE OF GREAT BRITAIN AND NORTHERN IRELAND) 16 April 1992 cited in the application see page 2, line 15 - page 13, line 8; claims	1,8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9300647
SA 72104

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 26/07/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9201056	23-01-92	FR-A-	2664614	17-01-92
		EP-A-	0538353	28-04-93
EP-A-0383509	22-08-90	CA-A-	2009708	13-08-90
		JP-A-	2295498	06-12-90
WO-A-9206198	16-04-92	AU-A-	8656691	28-04-92
		EP-A-	0551324	21-07-93
		AU-A-	8548991	28-04-92
		EP-A-	0551325	21-07-93
		WO-A-	9206197	16-04-92
WO-A-9206197	16-04-92	AU-A-	8548991	28-04-92
		EP-A-	0551325	21-07-93
		AU-A-	8656691	28-04-92
		EP-A-	0551324	21-07-93
		WO-A-	9206198	16-04-92

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/31, 15/62, C12Q 1/68
G01N 33/569, C07H 21/04

A1

(11) International Publication Number: WO 92/06198

(43) International Publication Date: 16 April 1992 (16.04.92)

(21) International Application Number: PCT/GB91/01691

(22) International Filing Date: 1 October 1991 (01.10.91)

(30) Priority data:

9021338.0	1 October 1990 (01.10.90)	GB
9022570.7	17 October 1990 (17.10.90)	GB

(71) Applicant *(for all designated States except US)*: THE MINISTER FOR AGRICULTURE, FISHERIES AND FOOD IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Horseferry Road, London SW1P 2AB (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : WOODWARD, Martin, John [GB/GB]; 23 Burnsall Close, Farnborough, Hampshire GU14 8NN (GB).

(74) Agent: LOCKWOOD, Peter, Brian; Patents 1A, MOD(PE), Room 2121, Empress State Building, Lillie Road, London SW6 1TR (GB).

(81) Designated States: AT (European patent), AU, BE (European patent), BG, BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, PL, RO, SE (European patent), SU+,US.

Published
With international search report.

(54) Title: SALMONELLA POLYNUCLEOTIDE SEQUENCE

(57) Abstract

DNA comprising a sequence characteristic of certain serotypes of the genus Salmonella is provided and used as polymerase chain reaction and hybridization targets for the identification of said serotypes. The DNA, in recombinant form, e.g. as plasmids, is used to transform suitable host cells to make them capable of expressing amino acid sequences characteristic of said serotypes. Test kits are provided comprising probes targeted at the characteristic sequences and amino acid sequences expressed by the transformants may also be used in immunological test kits for the serotypes.

+ See back of page

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

SALMONELLA POLYNUCLEOTIDE SEQUENCE

This invention relates to polynucleotides (DNA) comprising a sequence characteristic of certain serotypes of the genus Salmonella; to the use of sequences comprising the characteristic sequence as polymerase chain reaction and hybridization targets for the identification of said serotypes and to test kits for this; to the use of polynucleotides comprising the sequence to transform suitable host cells to make them capable of expressing amino acid sequences characteristic of said strains; to said amino acid sequence when so expressed and kits containing them; and to plasmids and transformed cells containing said polynucleotide sequences.

Organisms of the genus Salmonella, in particular S. enteritidis, S. dublin and S. typhimurium are responsible for infective food poisoning caused by their ingestion in contaminated food. Infection with Salmonella may also occur as a result of contact with contaminated materials. Once ingested, Salmonella is able to establish itself in the gut and multiply rapidly, resulting in the appearance of clinical symptoms several days after the initial ingestion.

It is therefore highly desirable to provide test methods by means of which Salmonella organisms may be detected. In recent years immunological tests have been devised in which specific antibodies, particularly monoclonal antibodies ("MABs"), to specific antigens are raised and which by exploitation of the antigen - antibody specific binding reaction the presence of the antigen can be detected. Such tests are fast and very specific.

It is known that Salmonella organisms have fimbria like structures on their surface (Duguid; J. P and R. R. Gillies (1958) J. Pathol. Bacteriol. 75:519-520) and published evidence (Clegg, S., and G. F. Gerlach (1987) J. Bacteriol. 169:934-938.) suggests that there are antigenically distinct types of fimbriae, ie. possessing specific epitopes on the fimbrial antigens. The possibility of immunogenic

tests for Salmonella, at least S. enteritidis, based upon these fimbrial antigens has been suggested (MAFF, Central Veterinary Laboratory "Animal Health" (1989):33). Methods of raising MABs to antigens on the surface of micro-organisms such as Salmonella are generally known.

Unfortunately known methods for raising antibodies to Salmonella surface antigens only go part way toward providing an immunological test for Salmonella. The basis of all these tests is to isolate micro-organisms from a sample suspected of harbouring Salmonella, then to grow the micro-organisms in vitro in a suitable culture medium until a quantity of the Salmonella sufficient to detect by such a test is believed to be present in the medium, and then applying the test. A problem occurs in that although Salmonella micro-organisms produce their fimbrial antigen when they grow in vivo, eg. in the gut, in animal tissues or fluids, in food products and in some natural environments, many of the fimbrial antigens are not produced when they are grown in vitro.

The present inventors have determined the polynucleotide sequence responsible for producing a characteristic fimbrial antigen, Salmonella enteritidis fimbrial antigen (SEFA). SEFA has an amino acid sequence forming an epitope on the fimbria 'in vivo' which is specifically found encoded by the DNA of the species S. enteritidis and some strains of the species S. dublin and S. Moscow but which is apparently absent in virtually all other serotypes. The identification and recognition of the significance of this sequence provides the basis for a number of determinative tests for the presence of the particular organisms or DNA/RNA derived from them and provides a method for production of transformed organisms capable of expressing SEFA or epitopic parts of SEFA.

The amino acid sequence of SEFA is provided below; it is of course to be expected that allelic variation will occur in some organisms.

AMINO ACID SEQUENCE OF SALMONELLA ENTERITIDIS FIMBRIAL ANTIGEN

M L I V D F W R F C N M R K S A S A V A V L A L I A C G S A H A A G F
V G N K A E V Q A A V T I A A Q N T T S A N W S Q D P G F T G P A V A
A G Q K V G T L S I T A T G P H N S V S I A G K G A S V S G G V A T V
P F V D G Q G Q P V F R G R I Q G A N I N D Q A N T G I D G L A G W R
V A S S Q E T L N V P V T T F G K S T L P A G T F T A T F Y V Q Q Y Q
N

The codes above are standard codes, read amino-terminal to carboxy -terminal, left to right, M to N, according to the following key:

Amino acid

Alanine	A	Lysine	K	Arginine	R
Methionine	M	Asparagine	N	Phenylalanine	F
Aspartic acid	D	Proline	P	Cysteine	C
Pyroglutamyl	*E	Glutamic acid	E	Serine	S
Glutamine	Q	Threonine	T	Glycine	G
Tryptophan	W	Histidine	H	Tyrosine	Y
Isoleucine	I	Valine	V	Leucine	L

Thus in its broadest form the present invention relates to DNA which forms all or part of the coding sequence for the SEFA sequence above or to allelic variants of that sequence, which carry the codons for its characteristic epitopes.

A first preferred aspect of the present invention provides recombinant DNA comprising the sequences I and II:

Sequence I

```
5'-  G CTCAGAATAC AACATCAGCC  AACTGGAGTC  AGGAT  -3'
3'-  C GAGTCTTATG TTGTAGTCGG  TTGACCTCAG  TCCTA  -5'
              230         240         250
```

Sequence II

```
5'-  CCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT
3'-  GGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA
       260         270         280         290         300

ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT
TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA
      310         320         330         340         350         360

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTT -3'
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAA -5'
      370         380         390         400         410
```

sequences degenerately equivalent thereto, or sequences encoding for allelic variants of the part of SEFA for which the sequences I and II encode.

The numerals below each ten base pair sequence in sequence I and II above are those designating the position of the individual base pairs in a larger characteristic sequence that comprises the entire SEFA antigen coding polynucleotide sequence.

By 'degenerately equivalent' is meant that substitute codons are present, these being codons which though they differ in their nucleotide base sequence from the codons identified in sequences I and II above, still code for the same amino acid, as will be understood by a man skilled in the art.

Preferred recombinant DNA of the invention, comprising sequences I and II, is that comprising sequences III and IV:

Sequence III

```
          5'- ATGCTAAT AGTTGATTTT TGGAGATTTT GTAATATGCG TAAATCAGCA
          3'- TACGATTA TCAACTAAAA ACCTCTAAAA CATTATACGC ATTTAGTCGT
                   80         90        100        110        120

TCTGCAGTAG CAGTTCTTGC TTTAATTGCA TGTGGCAGTG CCCACGCAGC TGGCTTTGTT
AGACGTCATC GTCAAGAACG AAATTAACGT ACACCGTCAC GGGTGCGTCG ACCGAAACAA
       130        140        150        160        170        180

GGTAACAAAG CAGAGGTTCA GGCAGCGGTT ACTATTGCAG CTCAGAATAC AACATCAGCC
CCATTGTTTC GTCTCCAAGT CCGTCGCCAA TGATAACGTC GAGTCTTATG TTGTAGTCGG
       190        200        210        220        230        240

AACTGGAGTC AGGAT -3'
TTGACCTCAG TCCTA -5'
       250
```

Sequence IV

```
          5'-  CCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT
          3'-  GGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA
                  260        270        280        290        300
```

```
ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT
TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA
       310         320         330         340         350         360

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTTC
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAAG
       370         380         390         400         410         420

CGTGGGCGTA TTCAGGGAGC CAATATTAAT GACCAAGCAA ATACTGGAAT TGACGGGCTT
GCACCCGCAT AAGTCCCTCG GTTATAATTA CTGGTTCGTT TATGACCTTA ACTGCCCGAA
       430         440         450         460         470         480

GCAGGTTGGC GAGTTGCCAG CTCTCAAGAA ACGCTAAATG TCCCTGTCAC AACCTTTGGT
CGTCCAACCG CTCAACGGTC GAGAGTTCTT TGCGATTTAC AGGGACAGTG TTGGAAACCA
       490         500         510         520         530         540

AAATCGACCC TGCCAGCAGG TACTTTCACT GCGACCTTCT ACGTTCAGCA GTATCAAAAC -3'
TTTAGCTGGG ACGGTCGTCC ATGAAAGTGA CGCTGGAAGA TGCAAGTCGT CATAGTTTTG -5'
       550         560         570         580         590         600
```

sequences degenerately equivalent thereto or sequences which encode for allelic variants of SEFA.

The significance of sequences III and IV is that when they run contiguously together, such that the -3' end of the top strand of sequence III is immediately followed by the top strand 5'- end of sequence IV, they consist of the polynucleotide sequence that encodes the amino acid sequence for SEFA (said upper strand).

Thus polynucleotide sequence encoding SEFA is on the upper strand as shown above beginning ATGCTAATAG on III and ending GTATCAAAAC on

IV. Further sequences which comprise suitable flanking sequences for control of amino acid sequence expression may be produced by genetic engineering techniques from this continuous sequence.

The invention further provides recombinant DNA comprising sequence III and IV, in the form of that comprising sequences V and VI:

Sequence V

```
5'- GATCCTTGTT TTTTTTCTTA AATTTTTAAA ATGGCGTGAG TATATTAGCA TCCGCACAGA
3'- CTAGGAACAA AAAAAAGAAT TTAAAAATTT TACCGCACTC ATATAATCGT AGGCGTGTCT
          10        20        30        40        50        60

    TAAATTGTGC GAATGCTAAT AGTTGATTTT TGGAGATTTT GTAATATGCG TAAATCAGCA
    ATTTAACACG CTTACGATTA TCAACTAAAA ACCTCTAAAA CATTATACGC ATTTAGTCGT
          70        80        90       100       110       120

    TCTGCAGTAG CAGTTCTTGC TTTAATTGCA TGTGGCAGTG CCCACGCAGC TGGCTTTGTT
    AGACGTCATC GTCAAGAACG AAATTAACGT ACACCGTCAC GGGTGCGTCG ACCGAAACAA
          130       140       150       160       170       180

    GGTAACAAAG CAGAGGTTCA GGCAGCGGTT ACTATTGCAG CTCAGAATAC AACATCAGCC
    CCATTGTTTC GTCTCCAAGT CCGTCGCCAA TGATAACGTC GAGTCTTATG TTGTAGTCGG
          190       200       210       220       230       240

    AACTGGAGTC AGGAT -3'
    TTGACCTCAG TCCTA -5'
          250
```

Sequence VI

```
                5'- CCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT
                3'- GGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA
                     260        270        280        290        300

ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT
TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA
      310        320        330        340        350        360

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTTC
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAAG
      370        380        390        400        410        420

CGTGGGCGTA TTCAGGGAGC CAATATTAAT GACCAAGCAA ATACTGGAAT TGACGGGCTT
GCACCCGCAT AAGTCCCTCG GTTATAATTA CTGGTTCGTT TATGACCTTA ACTGCCCGAA
      430        440        450        460        470        480

GCAGGTTGGC GAGTTGCCAG CTCTCAAGAA ACGCTAAATG TCCCTGTCAC AACCTTTGGT
CGTCCAACCG CTCAACGGTC GAGAGTTCTT TGCGATTTAC AGGGACAGTG TTGGAAACCA
      490        500        510        520        530        540

AAATCGACCC TGCCAGCAGG TACTTTCACT GCGACCTTCT ACGTTCAGCA GTATCAAAAC
TTTAGCTGGG ACGGTCGTCC ATGAAAGTGA CGCTGGAAGA TGCAAGTCGT CATAGTTTTG
      550        560        570        580        590        600

TAATTTAATT TAAACTTTAT AAATGCCCTC AATATGAGCG AGTTTGGATA ATTTTATTAT
ATTAAATTAA ATTTGAAATA TTTACGGGAG TTATACTCGC TCAAACCTAT TAAAATAATA
      610        620        630        640        650        660
```

TTTAAAAATA TCTATTTTGA ATAGATAGGT TTTATGCTTC CATGCAAAAA CTTAAAGAGG
AAATTTTTAT AGATAAAACT TATCTATCCA AAATACGAAG GTACGTTTTT GAATTTCTCC
670 680 690 700 710 720

GATTATGTAT ATTTTGAATA AATTTATACG TAGAACTGTT ATCTTTTTCC TTTTTTTTGC
CTAATACATA TAAAACTTAT TTAAATATGC ATCTTGACAA TAGAAAAAGG AAAAAAAACG
730 740 750 760 770 780

TACCTTCCAA TTGCTTCTTC GGAAAGTAAA AAAATTGAGC AACCATTATT AACACAAAAA
ATGGAAGGTT AACGAAGAAG CCTTTCATTT TTTTAACTCG TTGGTAATAA TTGTGTTTTT
790 800 810 820 830 840

TATTATGGCC TAAGATTGGG CACTACACGT GTTATTTATA AAGAAGATGC TCCATCAACA
ATAATACCGG ATTCTAACCC GTGATGTGCA CAATAAATAT TTCTTCTACG AGGTAGTTGT
850 860 870 880 890 900

AGTTTTTGGA TTATGAATGA AAAAGAATAT CCAATCCTTG TTCAAACTCA AGTATATAAT
TCAAAAACCT AATACTTACT TTTTCTTATA GGTTAGGAAC AAGTTTGAGT TCATATATTA
910 920 930 940 950 960

GATGATAAAT CATCAAAAGC TCCATTTATT GTAACACCAC CTATTTTGAA AGTTGAAAGT
CTACTATTTA GTAGTTTTCG AGGTAAATAA CATTGTGGTG GATAAAACTT TCAACTTTCA
970 980 990 1000 1010 1020

AATGCGCGAA CAAGATTGAA GGTAATACCA ACAAGTAATC TATTCAATAA AAATGAGGAG
TTACGCGCTT GTTCTAACTT CCATTATGGT TGTTCATTAG ATAAGTTATT TTTACTCCTC
1030 1040 1050 1060 1070 1080

```
TCTTTGTATT GGTTGTGTGT AAAAGGAGTC CCACCACTAA ATGATAATGA AAGCAATAAT
AGAAACATAA CCAACACACA TTTTCCTCAG GGTGGTGATT TACTATTACT TTCGTTATTA
      1090       1100       1110       1120       1130       1140

AAAAACAACA TAACTACGAA TCTTAATGTG AATGTGGTTA CGAATAGTTG TATTAAATTA
TTTTTGTTGT ATTGATGCTT AGAATTACAC TTACACCAAT GCTTATCAAC ATAATTTAAT
      1150       1160       1170       1180       1190       1200

ATTTATAGGC CTAAAACTAT AGACTTAACG ACAATGGAGA TTGCAGATAA ATTAAAGTTA
TAAATATCCG GATTTTGATA TCTGAATTGC TGTTACCTCT AACGTCTATT TAATTTCAAT
      1210       1220       1230       1240       1250       1260

GAGAGAAAAG GAAATAGTAT AGTTATAAAG AATCCAACAT CATCATATGT GAATATTGCA
CTCTCTTTTC CTTTATCATA TCAATATTTC TTAGGTTGTA GTAGTATACA CTTATAACGT
      1270       1280       1290       1300       1310       1320

AATATTAAAT CTGGTAATTT AAGTTTTAAT ATTCCAAATG GATATATTGA GCCATTTGGA
TTATAATTTA GACCATTAAA TTCAAAATTA TAAGGTTTAC CTATATAACT CGGTAAACCT
      1330       1340       1350       1360       1370       1380

TATGCTCAAT TACCTGGTGG AGTACATAGT AAAATAACTT TGACTATTTT GGATGATAAC
ATACGAGTTA ATGGACCACC TCATGTATCA TTTTATTGAA ACTGATAAAA CCTACTATTG
      1390       1400       1410       1420       1430       1440

GGCGCTGAAA TTATAAGAGA ATTATTAGTT TAAGGTGTAA AACAAATGAA GAAAACCACA
CCGCGACTTT AATATTCTCT TAATAATCAA ATTCCACATT TTGTTTACTT CTTTTGGTGT
      1450       1460       1470       1480       1490       1500
```

```
ATTACTCTAT TTGTTTTAAC CAGTGTATTT CACTCTGGAA ATGTTTTCTC CAGACAATAT
TAATGAGATA AACAAAATTG GTCACATAAA GTGAGACCTT TACAAAAGAG GTCTGTTATA
      1510       1520       1530       1540       1550       1560

AATTTCGACT ATGGAAGTTT GAGTCTTCTC CCGGTGAGAA TGCATCTTTT CTAAGTGTTG
TTAAAGCTGA TACCTTCAAA CTCAGAAGAG GGCCACTCTT ACGTAGAAAA GATTCACAAC
      1570       1580       1590       1600       1610       1620

AAACGCTTCC CTGGTAATTA TGTTGTTGAT GTATATTTGA ATAATCAGTT AAAAGAAACT
TTTGCGAAGG GACCATTAAT ACAACAACTA CATATAAACT TATTAGTCAA TTTTCTTTGA
      1630       1640       1650       1660       1670       1680

ACTGAGTTGT ATTTCAAATC AATGACTCAG ACTCTAGAAC CATGCTTAAC AAAAGAAAAA
TGACTCAACA TAAAGTTTAG TTACTGAGTC TGAGATCTTG GTACGAATTG TTTTCTTTTT
      1690       1700       1710       1720       1730       1740

CTTATAAAGT ATGGGATCGC CATCCAGGAG CTTCATGGGT TGCAGTTTGA TAATGAACAA
GAATATTTCA TACCCTAGCG GTAGGTCCTC GAAGTACCCA ACGTCAAACT ATTACTTGTT
      1750       1760       1770       1780       1790       1800

TGCGTTCTCT TAGAGCATTC TCCTCTTTAA ATATACTTAT AACGCGGCTA ACCAAAGTTT
ACGCAAGAGA ATCTCGTAAG AGGAGAAATT TATATGAATA TTGCGCCGAT TGGTTTCAAA
      1810       1820       1830       1840       1850       1860

GCTTTTAAAT GCACCATCTA AAATTCTATC TCCAATAGAC AGTGAAATTG CTGATGAAAA
CGAAAATTTA CGTGGTAGAT TTTAAGATAG AGGTTATCTG TCACTTTAAC GACTACTTTT
      1870       1880       1890       1900       1910       1920
```

```
TATCTGGGAT GATGGCATTA ACGCTTTTCT TTTAAATTAC AGAGCTTAAT TATTTGCATT
ATAGACCCTA CTACCGTAAT TGCGAAAAGA AAATTTAATG TCTCGAATTA ATAAACGTAA
      1930        1940        1950        1960        1970        1980

CTAAGGTTGG AGGAGAGAGA TTCATACTTT GGTCAAATTC AACCTTGGTT TTAATTTTGG
GATTCCAACC TCCTCTCTCT AAGTATGAAA CCAGTTTAAG TTGGAACCAA AATTAAAACC
      1990        2000        2010        2020        2030        2040

TCCCTGGCGG CTAAGGAATC TATCATCTTG GCAAAACTTG TCAAGCGAAA AAAAATTTGA
AGGGACCGCC GATTCCTTAG ATAGTAGAAC AGTTTTGAAC AGTTCGCTTT TTTTTAAACT
      2050        2060        2070        2080        2090        2100

ATCAGCATAT ATTTATGCTG AGCGAGGTTT AAAAAAAATA AAGAGCAAAC TAACAGTTGG
TAGTCGTATA TAAATACGAC TCGCTCCAAA TTTTTTTTAT TTCTCGTTTG ATTGTCAACC
      2110        2120        2130        2140        2150        2160

GGACAAATAT ACCAGTGCAG ATTTATTCGA TAGCGTACCA TTTAGAGGCT TTTCTTTAAA
CCTGTTTATA TGGTCACGTC TAAATAAGCT ATCGCATGGT AAATCTCCGA AAAGAAATTT
      2170        2180        2190        2200        2210        2220

TAAAGATGAA AGTATGATAC CTTTCTCACA GAGAACATAT TATCCAACAA TACGTGGTAT
ATTTCTACTT TCATACTATG GAAAGAGTGT CTCTTGTATA ATAGGTTGTT ATGCACCATA
      2230        2240        2250        2260        2270        2280

TGCGAAAACC AATGCGACTG TAGAAGTAAG ACAAAATGGA TACTTGATAT ATTCTACTTC
ACGCTTTTGG TTACGCTGAC ATCTTCATTC TGTTTTACCT ATGAACTATA TAAGATGAAG
      2290        2300        2310        2320        2330        2340

AGTCCCCCCC GGGCAATTCG AGATAGGTAG AGAACAAATT GCTGATC -3'
TCAGGGGGGG CCCGTTAAGC TCTATCCATC TCTTGTTTAA CGACTAG -5'
      2350        2360        2370        2380
```

or sequences degeneratively equivalent thereto.

For the purposes of expressing SEFA polypeptide or epitopic parts thereof the paired sequences I and II; III and IV; or V and VI run contiguously with each other without intervening base pairs between the two, in each case. These contiguous sequences are designated sequence VII, VIII and IX respectively.

For the purpose of expressing SEFA it will be realised by the skilled man that all the sequences above may comprise degenerate codons instead of those listed above. It is not envisaged that such use will necessarily provide any advantage as preparation would be probably be more lengthy, but some transformed microorganisms may express SEFA more readily with certain codons in degenerate form suited to them.

The present invention provides novel recombinant plasmids, comprising the recombinant DNA comprising either paired sequences selected from I and II, III and IV, or V and VI or the contiguous sequences VII, VIII and IX, the degenerative or allelic equivalents of any of these; said plasmids being capable of expressing polypeptides characteristic of SEFA when used to transform suitable microorganisms.

These recombinant plasmids may then be used to transform a host, such as E coli or yeast, whereby use of cloning and selection methods provides clones which contain the particular sequence or suitably flanked antigen encoding portion having expression enabling sequences with it. Convenient tools for the selection of these clones are the aforementioned sequences themselves as modified in known ways to provide probes, ie. by radiolabelling. Such probe sequences are readily provided by use of the polymerase chain reaction on native SEFA sequence template or by DNA synthesizer techniques; radiolabelling being achieved using standard techiques to tag on ^{32}P.

Preferred microorganisms for transformation are E. coli and yeasts; a particularly preferred microorganism being E. coli DH5alpha. Thus preferred plasmids will be those known to the man skilled in the art as suitable for transforming such organisms. Particularly preferred plasmids are accordingly pBR322, pACYC184 and, most preferred, pUC18.

The polynucleotides sequences above may be combined with any of these known plasmids for the purposes of providing the novel plasmids of the invention. Particularly preferred will be plasmids into which polynucleotides consisting of the contiguous sequences VIII or IX have been inserted as these will be readily provided from cultured S. enteritidis or S. dublin by use of restriction endonucleases and encode for the entire SEFA amino acid sequence. In this respect use of antibodies targeted for SEFA allows facile recognition of transformed organisms which is particularly useful for selecting expressing organisms from a background population. Such antibodies are the subject of copending MAFF patent application (PCT GB 91-----, our reference P0958) of inventor C J Thorns).(See Tables I and II).

For example, the contiguous sequence IX may be blunt-ended using Klenow polymerase infilling and then ligated into a plasmid such as pUC18. Alternatively total genomic DNA is extracted from S. enteritidis or a strain of S. dublin possessing said fimbrial antigen, as determined using the monoclonal antibodies and techniques disclosed in the applicants copending application referred to above, and then partially digested with SauIIIA restriction endonuclease to leave large fragments, some of which contain the sequences referred to above, which are then ligated into the plasmid vectors above.

The vectors of the present invention have further utility in so far as the contiguous sequences VII, VIII and IX all comprise a single BamH1 restriction endonuclease recognition site into which foreign peptide encoding DNA may be ligated by which it is sited within the reading frame of the transformant transcription system. This site is

at the junction between the two sequences that make up the contiguous sequence; that occuring between base pair 255 and 256 in the numbering system applied at the bottom of each 10 base pairs above. Thus the present invention provides plasmids and transformants comprising the sequences I and II, or III and IV, or V and VI, or their degenerative or allelic equivalents, which have been augmented with further sequences. The invention provides a method for preparing these plasmid and transformants which inserts the further sequences into plasmids comprising the contiguous sequences VII, VIII or IX the at that BamH1 site.

Such augmented transformants are potentially capable of expression of mixed epitopic polypeptides comprising epitopes of SEFA together with further 'foreign' peptides. This opens the way to recombinantly produced peptides that are not easily expressed by other means. The fact that SEFA is a polypeptide that is passed to the exterior of the Salmonella cell of advantage in the recovery of such expressed polypeptides. The 'foreign' peptides may be further SEFA epitopes.

Thus the invention also provides micro-organisms, eg E.coli or yeasts, which have been transformed by insertion of one or more of the aforementioned sequences eg, by use of said plasmids.

Use of the micro-organisms provided by the invention gives a method of expression of the antigenic amino acid sequence SEFA referred to above and epitopic parts thereof which might be used as antigenic activity, that is having the ability to evoke production of antibodies in animal bodies.

In addition to use of the transformant expressed SEFA or epitopic parts thereof for immunological test purposes and kits for such, the recognition of the significance of the DNA sequences defined above provides methods of determination of DNA or RNA as being derived from the S. enteritidis or S. dublin serotypes in other, DNA/RNA based, tests.

TABLE I

264 Salmonella strains examined with monoclonal antibody MAB69/25

Serogroup	Serotype (No. strains tested)
B	S. agama (1)
	S. agona (1)
	S. bredeney (1)
	S. derby (1)
	S. heidelberg (1)
	S. indiana (1)
	S. reading (1)
	S. schwarzengrund (1)
	S. stanley (1)
	S. typhimurium (64)
C1	S. bareilly (1)
	S. infantis (1)
	S. lille (1)
	S. livingstone (1)
	S. mbandaka (1)
	S. montevideo (1)
	S. ohio (1)
	S. oranienburg (1)
	S. oslo (1)
	S. thompson (1)
	S. virchow (1)
C2	S. goldcoast (1)
	S. hadar (1)
	S. newport (1)
C3	S. albany (1)
	S. kentucky (2)
	S. tado (1)
D1	S. berta (1)
	S. canastel (1)
	S. dublin (36)
	S. durban (1)
	S. enteritidis (58)
D1	S. gallinarium (44)
	S. moscow (1)
	S. ouakam (1)
	S. panama (1)
	S. pullorum (3)
	S. wangata (1)
E1	S. anatum (1)
	S. give (1)
	S. lexington (1)
	S. london (1)
	S. meleagridis (1)
	S. nchanga (1)
	S. orion (1)
E2	S. binza (1)
	S. drypool (1)
	S. manila (1)
	S. newington (1)
E4	S. taksony (1)
	S. senftenberg (1)
F	S. aberdeen (1)
G1	S. havana (1)
	S. worthington (1)
G2	S. ajiobo (1)
	S. kedougou (1)
K	S. cerro (1)
N	S. urbana (1)
O	S. adelaide (1)
	S. ealing (1)
R	S. johannesburg (1)
S	S. offa (1)
T	S. gera (1)

TABLE II

Direct binding of MAB 69/25 to Salmonella strains

Serotype		Number Examined	Monoclonal antibody MAB 69/25 %bound
S. enteritidis	PT 1	2	56[a] (48-64)[b]
S. enteritidis	PT 4	22	57 (14-100)
S. enteritidis	PT 4 plasmid minus	6	57 (49-65)
S. enteritidis	PT 5	1	83
S. enteritidis	PT 6	1	57
S. enteritidis	PT 7	1	89 (85-93)
S. enteritidis	PT 8	12	53 (15-90)
S. enteritidis	PT 9	4	20 (17-23)
S. enteritidis	PT 11	7	50 (23-77)
S. enteritidis	PT 30	1	15
S. enteritidis	untypable	1	41
S. dublin		12	25 (9-40)
S. dublin		24	0
S. moscow		1	9
Other Salmonella strains[c]		169	0

[a] Mean percentage of antibody binding relative to binding to high control (see text)

[b] Range of binding

[c] Serotypes listed in Table I

PT = Phage type

The present invention further provides methods for determining the presence of microorganisms having DNA or RNA polynucleotide sequence encoding for SEFA or an epitopic part thereof, or such DNA or RNA itself, comprising:

(a) providing a sample suspected of containing said encoding polynucleotide sequence;

(b) determining the presence of said sequence by monitoring hybridization of SEFA targeted polynucleotide probes to it.

Such hybridization technique is carried out by methods that are now conventional in the art, using probes which are comprised of sequences complementary to a significant part of the target sequence and using temperature conditions suitable to achieved a desired stringency dependent on the degree of match of the probe to the target.

In a preferred form of this method the invention further provides methods for determining the presence of microorganisms having DNA or RNA polynucleotide sequence encoding for SEFA or an epitopic part thereof, or such DNA or RNA itself, comprising:

(a) providing a sample suspected of containing said encoding polynucleotide sequence;

(b) subjecting said sample to conditions under which polynucleotide sequences comprising sequences (I) and (II) are replicated by use of the polymerase chain reaction;

(c) determining the presence of any sequence produced.

Conveniently the sequence produced is detected, in both cases, by use of a hybridization probe suitably specific thereto which comprises any

of the aforementioned sequences, more specifically being one of the sequences in a suitably labelled form eg. being labelled in some way as will be known to a man skilled in the art. Most conveniently the label will incorporate radioactive phophorous (^{32}P). A preferred such method comprises a PCR step (b) which employs primer pairs comprising one primer selected from groups (A) and the other from group(B):

Group A:	Group B:
A1: 5' -GTGCGAATGCTAATAGTTGA- 3'	B1: 5' -AAAACAGGCTGTCCTTGTCCA- 3'
A2: 5' -TGCGTAAATCAGCATCTGCA- 3'	B2: 5' -TTAGCGTTTCTTGAGAGCTGG- 3'
A3: 5' -TCTGCAGTAGCAGTTCTTGC- 3'	B3: 5' -TTTTGATACTGCTGAACGTAG- 3'
A4: 5' -GCTCAGAATACAACATCAGCCAA- 3'	

The primers are numbered A1 to A4 and B1 to B3 for the purposes of identification later in this specification.

Any of the possible pairs selected in this way will identify the characteristic sequences VI, VII or IX sufficiently specifically enough for serotype determination purposes, ie: for determination of a Salmonella as being a SEFA encoding serotype and thus of one of the serotypes listed above.

As will be understood by a man skilled in the art, sequences which will specifically hybridize with sequence (VII) will include sequence (VII) itself, those having 75% or more, preferably 90% or more conformity to that sequence, and sequences comprising either strand of the two complementary sequences of any of these. Thus the step (c) of the method of this aspect of the invention may be carried out using a variety of hybridization probes that combine sufficiently

specifically with the characteristic 'target' sequence comprising sequence (VII). For most purposes the primer sequences selected from those of groups (A) and (B) will be sufficiently specific to give reliable determination of the characteristic sequence, especially if a different 'primer' sequence is used for the probe of step (c) than those used for step (b).

The step (b) is carried out using the enzyme Taq polymerase as is now conventional in the art. The necessary conditions are those as described in EP-A-0201184 or EP-A-0200362 (both Cetus Corpn.) In such reaction, the appropriate primers derived from the sequences act as initiators for synthesis of large quantities of DNA identical to, or substantially identical to the initial double stranded DNA sequence. In this way substantially larger quantities of the DNA sequence may be made from the small quantities which may be available by isolation from the S. enteritidis or S. dublin thus increasing the amount of sequence available to be detected. The mere opresence of increased amount of DNA may be used in this case to signify presence of target sequence.

The genetically transformed organisms of the invention and their use to produce SEFA and SEFA containing sequences of the invention will now be described by way of example only, the examples including use of the detection methods of the invention for confirming presence of transformants:

Example A. Preparation and cloning of S. enteritidis fimbrial antigen genes.

Step A1. Total genomic DNA was extracted from S. enteritidis using the method described in J B Goldberg & D E Ohman, (1984) J Bact 158 1115-1121.

Step A2. The DNA from step A1 was partially digested with SauIIIA restriction endonuclease to yield fragments with an size range between 5 and 10 kb. 2ug of genomic DNA in a Tris-HCl based buffer of pH 7.4 were mixed with 0.25 units of SauIIIA and incubated at 37°C.

Step A3. Cloning vector pUC18 was digested to completion with BamH1, giving compatible cohesive ends with SauIIIA, and was dephosphorylated with calf intestinal phosphatase.

Step A4. S. enteritidis DNA was ligated with vector pUC18 using T4 DNA ligase supplied by Bethesda Research Laboratories Life Technologies Inc. (Cat. No. 5224SB/SC). The supplier's instructions for use in ligation were followed.

Step A5. The recombinant plasmid from step A4 was used to transform commercially available E.coli DH5alpha supplied by Bethesda Labs (see above) as Library Efficiency (RTM) DH5alpha Competant Cells (Cat. No. 8263SA) using the supplier's instructions to produce a genomic library.

Step A6. Transformants were transferred to the surface of HYBOND-C filters by replica plating for Western Blotting. Standard Western Blotting procedures using the S. enteritidis fimbrial antigen specific monoclonal antibody MAB 69/25, derived by standard techniques from hybridoma cells deposited under Accession No.90101101 on 11 October 1990 at the European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 OJG, United Kingdom, as described and claimed in copending application No (PCT GB91 ;our ref P0958WOD), were done to identify transformant colonies expressing SEFA and thus containing the aforementioned sequences (VI), (VII) and (IX).

Step A7. The recombinant plasmids from fimbrial antigen positive transformants were extracted and used in confirmatory tests to prove

the insert encoded said fimbrial antigen.

At the end of stage A7 it is possible to probe the DNA of said transformants to show the presence of the sequences and then to analyse said sequence by known sequencing methods.

EXAMPLE B: Presentation of epitopes within the SEFA antigen by insertion of foreign DNA, in frame, into the SEFA encoding sequence.

As stated above, the present invention further provides the prospect of exploitation of the polynucleotide sequences of the present invention having with sequences encoding for desired foreign protein or peptide products to produce transformants having ability to secrete the desired product.

SB10 epitope of Mycobacterium bovis secreted antigen, MPB70 (Radford et al, (1990), J. Gen. Micro. 136: 265-272) consists of the amino acid sequence as encoded for below:

Q D P V	encoded amino acid
5'- CAG GAC CCG GTC -3'	coding/master strand
3'- GTC CTG GGC CAG -5'	complimentary strand

Synthetic oligonucleotides encompassing this sequence and providing BamH1 cohesive ends were made using an ABI PCR MATE EP model 391 DNA synthesizer following the manufacturer's methods. The oligonucleotides were as follows:

SB10.1	5'-	GAT CAG GAC CCG GTC GCT	-3'
SB10.2	3'-	TC CTG GGC CAG CGA CTA G	-5'

The two oligonucleotides, SB10.1 and SB10.2 were allowed to anneal to form a double stranded (duplex) molecule by heating to 95°C and then cooling to room temperature over a two hour period. Annealing was assessed by comparing rate of migration of the duplex molecule compared with the rate of migration of the two single oligonucleotides when run through 4% agarose in TBE buffer. A marginal retardation in migration rate was observed and suggested near 100% annealing.

A lambda EMBL library was prepared from S.enteritidis strain 1246 providing a 9 to 23 kilobase library which was probed with the SEFA sequence IX (consisting of sequences V and VI run contiguously). Hybridizing fragments were subcloned into pUC18 and a suitable vector comprising the SEFA antigen gene flanked by adjacent contiguous chromosomal DNA was selected on its ability to transform E.coliDH5 alpha to a SEFA expressing form: all general methods as conventional to the art (see eg. Maniatis).

The pUC18 vector so obtained was digested with BamH1 and agarose gel electrophoresis demonstrated that the DNA was cut once at the unique BamH1 site within the SEFA gene. Cut vector and duplex oligonucleotide (SB10.1 plus SB10.2) were mixed together (1:10 ratio) and ligated using T4 ligase (Life Technologies) using the manufacturers methods. The saturating amounts of duplex oligonucleotide increased rate of insertion and the lack of terminal phosphate groups on the duplex prevented multiple insertion. The ligated construct was designed to be as follows:

	Q	D	Q	D	P	V	A	D	P	amino acid
5'-	CAG	GAT	CAG	GAC	CCG	GTC	GCT	GAT	CCT -3'	coding/master strand
3'-	GTC	CTA	GTC	CTG	GGC	CAG	CGA	CTA	GGA -5'	complimentary strand

The ligated construct lacks the GGATCC BamH1 recognition sequence.

Thus prior to transforming the construct into E.coli DH5 alpha, the ligated DNA was cut with BamH1 to linearise any of the vector which lacked insert. The ligated DNA was then used to transform E.coli using standard procedures.

Recombinants were picked directly into a Polymerase Chain Reaction mixture in which the primers were designed to flank the insertion site to yield a product of 219 base pairs without insert or 237 base pairs with insert. PCR products were sized by gel lectrophoresis and those shown to be 237 base pairs were tested by digestion with BamH1 to ensure loss of the site.

A sample (8ul) was taken from the aqueous phase of the PCR reaction mixture and made 20ul by addition of HPLC grade water, X10 reaction buffer and 5U BamH1. The PCR product was digested for 3 hours at 37°C. Control experiments using the 219 base pair product were performed to demonstrate digestion. The entire reaction mixtures were loaded onto agarose gels and the DNA products resolved; those PCR products shown to be 237 base pairs did not cut with BamH1 giving evidence for insertion of the oligonucleotide duplex.

To confirm the presence of the insert and determine its orientation, PCR experiments were set up in which the primers were SB10.2 and a series of primers from primer group A above (see page 18) toward the proximal (5') end of the SEFA antigen gene. Of twelve recombinants tested, five gave the desired sized product and were, therefore, shown to have the insert in the correct orientation.

To confirm that the insert was encoding the SB10 epitope and was 'in frame' with the SEFA antigen sequence, double stranded DNA sequencing using standard protocols was done on the five positive clones identified above. The primers used were:

5'- TCTGCAGTAGCAGTTCTTGC -3' for the coding strand and

5'- AAAACAGGCTGTCCTTGTCCA -3' for the complimentary strand.

The DNA sequence of both strands across the insert site was established and was as predicted above.

E. coli recombinants harbouring the constructs, designated SEFA::SB10. 1 to 5 were tested immunologically for the production of SEFA. Western blots of whole E. coli cells harbouring each of the SEFA::SB10 constructs demonstrated the presence of a protein of about 15kDal (and a less intense protein band of about 18.5 kDal) when using anti-SEFA polyclonal and anti-SEFA monoclonal antibody 69/25. In control experiments, E. coli recombinants harbouring the vector gave protein bands of 14.5kDal and 18kDal in Western blot experiments using the same antibodies.

This data clearly demonstrates that the SEFA polynucleotide sequence may be modified to express additional amino acids within its primary structure without the loss of reactivity to one SEFA epitope specific antibody.

The complete sequence of the largest of the sequences of the invention, sequence IX, is given below with the sequences I, II, III, IV, V, VI, VII and VIII being indicated together with the probe sequences from probe groups A and B. These sequences are marked by reference to their 5' and 3' ends: eg. I-5', I-3' etc. The numbering given below each 10 base pairs of the sequences I to VI above being related to their positions in this sequence IX.

Sequence IX

V-5'

5'- GATCCTTGTT TTTTTTCTTA AATTTTTAAA ATGGCGTGAG TATATTAGCA TCCGCACAGA

3'- CTAGGAACAA AAAAAAGAAT TTAAAAATTT TACCGCACTC ATATAATCGT AGGCGTGTCT

10 20 30 40 50 60

A1-5' III-5' A1-3' A2-5'

TAAATTGTGC GAATGCTAAT AGTTGATTTT TGGAGATTTT GTAATATTGCG TAAATCAGCA

ATTTAACACG CTTACGATTA TCAACTAAAA ACCTCTAAAA CATTATACGC ATTTAGTCGT

70 80 90 100 110 120

A3-5'

A2-3' A3-3'

TCTGCAGTAG CAGTTCTTGC TTTAATTGCA TGTGGCAGTG CCCACGCAGC TGGCTTTGTT

AGACGTCATC GTCAAGAACG AAATTAACGT ACACCGTCAC GGGTGCGTCG ACCGAAACAA

130 140 150 160 170 180

A4-5'

GGTAACAAAG CAGAGGTTCA GGCAGCGGTT ACTATTGCAG CTCAGAATAC AACATCAGCC

CCATTGTTTC GTCTCCAAGT CCGTCGCCAA TGATAACGTC GAGTCTTATG TTGTAGTCGG

190 200 210 220 230 240

A4-3'

I,III and V -3':II,IV and VI-5'

AACTGGAGTC AGGATCCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT

TTGACCTCAG TCCTAGGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA

250 260 270 280 290 300

BamH1 site.

ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT

TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA

310 320 330 340 350 360

II-3'

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTTC
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAAG
370 380 390 400 410 420

B1-3' B1-5'

CGTGGGCGTA TTCAGGGAGC CAATATTAAT GACCAAGCAA ATACTGGAAT TGACGGGCTT
GCACCCGCAT AAGTCCCTCG GTTATAATTA CTGGTTCGTT TATGACCTTA ACTGCCCGAA
430 440 450 460 470 480

GCAGGTTGGC GAGTTGCCAG CTCTCAAGAA ACGCTAAATG TCCCTGTCAC AACCTTTGGT
CGTCCAACCG CTCAACGGTC GAGAGTTCTT TGCGATTTAC AGGGACAGTG TTGGAAACCA
490 500 510 520 530 540

B2-3' B2-5'

IV-3'

AAATCGACCC TGCCAGCAGG TACTTTCACT GCGACCTTCT ACGTTCAGCA GTATCAAAAC
TTTAGCTGGG ACGGTCGTCC ATGAAAGTGA CGCTGGAAGA TGCAAGTCGT CATAGTTTTG
550 560 570 580 590 600

B3-3' B3-5'

TAATTTAATT TAAACTTTAT AAATGCCCTC AATATGAGCG AGTTTGGATA ATTTTATTAT
ATTAAATTAA ATTTGAAATA TTTACGGGAG TTATACTCGC TCAAACCTAT TAAAATAATA
610 620 630 640 650 660

TTTAAAAATA TCTATTTTGA ATAGATAGGT TTTATGCTTC CATGCAAAAA CTTAAAGAGG
AAATTTTTAT AGATAAAACT TATCTATCCA AAATACGAAG GTACGTTTTT GAATTTCTCC
670 680 690 700 710 720

GATTATGTAT ATTTTGAATA AATTTATACG TAGAACTGTT ATCTTTTTCC TTTTTTTTGC
CTAATACATA TAAAACTTAT TTAAATATGC ATCTTGACAA TAGAAAAAGG AAAAAAAACG
730 740 750 760 770 780

```
TACCTTCCAA TTGCTTCTTC GGAAAGTAAA AAAATTGAGC AACCATTATT AACACAAAAA
ATGGAAGGTT AACGAAGAAG CCTTTCATTT TTTTAACTCG TTGGTAATAA TTGTGTTTTT
       790        800        810        820        830        840

TATTATGGCC TAAGATTGGG CACTACACGT GTTATTTATA AAGAAGATGC TCCATCAACA
ATAATACCGG ATTCTAACCC GTGATGTGCA CAATAAATAT TTCTTCTACG AGGTAGTTGT
       850        860        870        880        890        900

AGTTTTTGGA TTATGAATGA AAAAGAATAT CCAATCCTTG TTCAAACTCA AGTATATAAT
TCAAAAACCT AATACTTACT TTTTCTTATA GGTTAGGAAC AAGTTTGAGT TCATATATTA
       910        920        930        940        950        960

GATGATAAAT CATCAAAAGC TCCATTTATT GTAACACCAC CTATTTTGAA AGTTGAAAGT
CTACTATTTA GTAGTTTTCG AGGTAAATAA CATTGTGGTG GATAAAACTT TCAACTTTCA
       970        980        990       1000       1010       1020

AATGCGCGAA CAAGATTGAA GGTAATACCA ACAAGTAATC TATTCAATAA AAATGAGGAG
TTACGCGCTT GTTCTAACTT CCATTATGGT TGTTCATTAG ATAAGTTATT TTTACTCCTC
      1030       1040       1050       1060       1070       1080

TCTTTGTATT GGTTGTGTGT AAAAGGAGTC CCACCACTAA ATGATAATGA AAGCAATAAT
AGAAACATAA CCAACACACA TTTTCCTCAG GGTGGTGATT TACTATTACT TTCGTTATTA
      1090       1100       1110       1120       1130       1140

AAAAACAACA TAACTACGAA TCTTAATGTG AATGTGGTTA CGAATAGTTG TATTAAATTA
TTTTTGTTGT ATTGATGCTT AGAATTACAC TTACACCAAT GCTTATCAAC ATAATTTAAT
      1150       1160       1170       1180       1190       1200
```

ATTTATAGGC CTAAAACTAT AGACTTAACG ACAATGGAGA TTGCAGATAA ATTAAAGTTA
TAAATATCCG GATTTTGATA TCTGAATTGC TGTTACCTCT AACGTCTATT TAATTTCAAT
1210 1220 1220 1240 1250 1260

GAGAGAAAAG GAAATAGTAT AGTTATAAAG AATCCAACAT CATCATATGT GAATATTGCA
CTCTCTTTTC CTTTATCATA TCAATATTTC TTAGGTTGTA GTAGTATACA CTTATAACGT
1270 1280 1290 1300 1310 1320

AATATTAAAT CTGGTAATTT AAGTTTTAAT ATTCCAAATG GATATATTGA GCCATTTGGA
TTATAATTTA GACCATTAAA TTCAAAATTA TAAGGTTTAC CTATATAACT CGGTAAACCT
1330 1340 1350 1360 1370 1380

TATGCTCAAT TACCTGGTGG AGTACATAGT AAAATAACTT TGACTATTTT GGATGATAAC
ATACGAGTTA ATGGACCACC TCATGTATCA TTTTATTGAA ACTGATAAAA CCTACTATTG
1390 1400 1410 1420 1430 1440

GGCGCTGAAA TTATAAGAGA ATTATTAGTT TAAGGTGTAA AACAAATGAA GAAAA[illegible]CACA
CCGCGACTTT AATATTCTCT TAATAATCAA ATTCCACATT TTGTTTACTT CTTTTGGTGT
1450 1460 1470 1480 1490 1500

ATTACTCTAT TTGTTTTAAC CAGTGTATTT CACTCTGGAA ATGTTTTCTC CAGACAATAT
TAATGAGATA AACAAAATTG GTCACATAAA GTGAGACCTT TACAAAAGAG GTCTGTTATA
1510 1520 1530 1540 1550 1560

AATTTCGACT ATGGAAGTTT GAGTCTTCTC CCGGTGAGAA TGCATCTTTT CTAAGTGTTG
TTAAAGCTGA TACCTTCAAA CTCAGAAGAG GGCCACTCTT ACGTAGAAAA GATTCACAAC
1570 1580 1590 1600 1610 1620

AAACGCTTCC CTGGTAATTA TGTTGTTGAT GTATATTTGA ATAATCAGTT AAAAGAAACT
TTTGCGAAGG GACCATTAAT ACAACAACTA CATATAAACT TATTAGTCAA TTTTCTTTGA
1630 1640 1650 1660 1670 1680

ACTGAGTTGT ATTTCAAATC AATGACTCAG ACTCTAGAAC CATGCTTAAC AAAAGAAAAA
TGACTCAACA TAAAGTTTAG TTACTGAGTC TGAGATCTTG GTACGAATTG TTTTCTTTTT
1690 1700 1710 1720 1730 1740

CTTATAAAGT ATGGGATCGC CATCCAGGAG CTTCATGGGT TGCAGTTTGA TAATGAACAA
GAATATTTCA TACCCTAGCG GTAGGTCCTC GAAGTACCCA ACGTCAAACT ATTACTTGTT
1750 1760 1770 1780 1790 1800

TGCGTTCTCT TAGAGCATTC TCCTCTTTAA ATATACTTAT AACGCGGCTA ACCAAAGTTT
ACGCAAGAGA ATCTCGTAAG AGGAGAAATT TATATGAATA TTGCGCCGAT TGGTTTCAAA
1810 1820 1830 1840 1850 1860

GCTTTTAAAT GCACCATCTA AAATTCTATC TCCAATAGAC AGTGAAATTG CTGATGAAAA
CGAAAATTTA CGTGGTAGAT TTTAAGATAG AGGTTATCTG TCACTTTAAC GACTACTTTT
1870 1880 1890 1900 1910 1920

TATCTGGGAT GATGGCATTA ACGCTTTTCT TTTAAATTAC AGAGCTTAAT TATTTGCATT
ATAGACCCTA CTACCGTAAT TGCGAAAAGA AAATTTAATG TCTCGAATTA ATAAACGTAA
1930 1940 1950 1960 1970 1980

CTAAGGTTGG AGGAGAGAGA TTCATACTTT GGTCAAATTC AACCTTGGTT TTAATTTTGG
GATTCCAACC TCCTCTCTCT AAGTATGAAA CCAGTTTAAG TTGGAACCAA AATTAAAACC
1990 2000 2010 2020 2030 2040

```
TCCCTGGCGG CTAAGGAATC TATCATCTTG GCAAAACTTG TCAAGCGAAA AAAAATTTGA
AGGGACCGCC GATTCCTTAG ATAGTAGAAC AGTTTTGAAC AGTTCGCTTT TTTTTAAACT
      2050      2060      2070      2080      2090      2100

ATCAGCATAT ATTTATGCTG AGCGAGGTTT AAAAAAAATA AAGAGCAAAC TAACAGTTGG
TAGTCGTATA TAAATACGAC TCGCTCCAAA TTTTTTTTAT TTCTCGTTTG ATTGTCAACC
      2110      2120      2130      2140      2150      2160

GGACAAATAT ACCAGTGCAG ATTTATTCGA TAGCGTACCA TTTAGAGGCT TTTCTTTAAA
CCTGTTTATA TGGTCACGTC TAAATAAGCT ATCGCATGGT AAATCTCCGA AAAGAAATTT
      2170      2180      2190      2200      2210      2220

TAAAGATGAA AGTATGATAC CTTTCTCACA GAGAACATAT TATCCAACAA TACGTGGTAT
ATTTCTACTT TCATACTATG GAAAGAGTGT CTCTTGTATA ATAGGTTGTT ATGCACCATA
      2230      2240      2250      2260      2270      2280

TGCGAAAACC AATGCGACTG TAGAAGTAAG ACAAAATGGA TACTTGATAT ATTCTACTTC
ACGCTTTTGG TTACGCTGAC ATCTTCATTC TGTTTTACCT ATGAACTATA TAAGATGAAG
      2290      2300      2310      2320      2330      2340

                                                VI-3'
AGTCCCCCCC GGGCAATTCG AGATAGGTAG AGAACAAATT GCTGATC -3'
TCAGGGGGGG CCCGTTAAGC TCTATCCATC TCTTGTTTAA CGACTAG -5'
      2350      2360      2370      2380
```

CLAIMS

1. Recombinant DNA encoding for the Salmonella enteritidis fimbrial antigen amino acid sequence:

M L I V D F W R F C N M R K S A S A V A V L A L I A C G S A H A A G F
V G N K A E V Q A A V T I A A Q N T T S A N W S Q D P G F T G P A V A
A G Q K V G T L S I T A T G P H N S V S I A G K G A S V S G G V A T V
P F V D G Q G Q P V F R G R I Q G A N I N D Q A N T G I D G L A G W R
V A S S Q E T L N V P V T T F G K S T L P A G T F T A T F Y V Q Q Y Q
N

for an epitopic part thereof or for alleles of either.

2. Recombinant DNA as claimed in Claim 1 comprising the sequences I and II:

Sequence I

```
5'-  G CTCAGAATAC AACATCAGCC  AACTGGAGTC  AGGAT  -3'
3'-  C GAGTCTTATG TTGTAGTCGG  TTGACCTCAG  TCCTA  -5'
              230         240         250
```

Sequence II

```
5'-  CCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT
3'-  GGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA
       260         270         280         290         300

ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT
TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA
      310         320         330         340         350         360
```

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTT -3'
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAA -5'
370 380 390 400 410

sequences degenerately equivalent thereto, or sequences encoding for allelic variants of the part of SEFA for which the sequences I and II encode.

3. Recombinant DNA as claimed in Claim 1 or 2 wherein the sequence comprising sequences I and II comprises sequences III and IV:

Sequence III

5'- ATGCTAAT AGTTGATTTT TGGAGATTTT GTAATATGCG TAAATCAGCA
3'- TACGATTA TCAACTAAAA ACCTCTAAAA CATTATACGC ATTTAGTCGT
80 90 100 110 120

TCTGCAGTAG CAGTTCTTGC TTTAATTGCA TGTGGCAGTG CCCACGCAGC TGGCTTTGTT
AGACGTCATC GTCAAGAACG AAATTAACGT ACACCGTCAC GGGTGCGTCG ACCGAAACAA
130 140 150 160 170 180

GGTAACAAAG CAGAGGTTCA GGCAGCGGTT ACTATTGCAG CTCAGAATAC AACATCAGCC
CCATTGTTTC GTCTCCAAGT CCGTCGCCAA TGATAACGTC GAGTCTTATG TTGTAGTCGG
190 200 210 220 230 240

AACTGGAGTC AGGAT -3'
TTGACCTCAG TCCTA -5'
250

Sequence IV

```
5'-  CCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT
3'-  GGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA
       260        270        280        290        300

ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT
TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA
      310        320        330        340        350        360

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTTC
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAAG
      370        380        390        400        410        420

CGTGGGCGTA TTCAGGGAGC CAATATTAAT GACCAAGCAA ATACTGGAAT TGACGGGCTT
GCACCCGCAT AAGTCCCTCG GTTATAATTA CTGGTTCGTT TATGACCTTA ACTGCCCGAA
      430        440        450        460        470        480

GCAGGTTGGC GAGTTGCCAG CTCTCAAGAA ACGCTAAATG TCCCTGTCAC AACCTTTGGT
CGTCCAACCG CTCAACGGTC GAGAGTTCTT TGCGATTTAC AGGGACAGTG TTGGAAACCA
      490        500        510        520        530        540

AAATCGACCC TGCCAGCAGG TACTTTCACT GCGACCTTCT ACGTTCAGCA GTATCAAAAC -3'
TTTAGCTGGG ACGGTCGTCC ATGAAAGTGA CGCTGGAAGA TGCAAGTCGT CATAGTTTTG -5'
      550        560        570        580        590        600
```

sequences degenerately equivalent thereto or sequences which encode for allelic variants of SEFA.

4. Recombinant DNA as claimed in any one of the preceding claims wherein suitable flanking sequences for control of amino acid sequence expression are provided.

5. Recombinant DNA as claimed in any one of the preceding claims wherein the sequences I and II are provided in sequences comprising sequences V and VI respectively:

Sequence V

```
5'- GATCCTTGTT TTTTTTCTTA AATTTTTAAA ATGGCGTGAG TATATTAGCA TCCGCACAGA
3'- CTAGGAACAA AAAAAAGAAT TTAAAAATTT TACCGCACTC ATATAATCGT AGGCGTGTCT
          10         20         30         40         50         60

    TAAATTGTGC GAATGCTAAT AGTTGATTTT TGGAGATTTT GTAATATGCG TAAATCAGCA
    ATTTAACACG CTTACGATTA TCAACTAAAA ACCTCTAAAA CATTATACGC ATTTAGTCGT
          70         80         90        100        110        120

    TCTGCAGTAG CAGTTCTTGC TTTAATTGCA TGTGGCAGTG CCCACGCAGC TGGCTTTGTT
    AGACGTCATC GTCAAGAACG AAATTAACGT ACACCGTCAC GGGTGCGTCG ACCGAAACAA
         130        140        150        160        170        180

    GGTAACAAAG CAGAGGTTCA GGCAGCGGTT ACTATTGCAG CTCAGAATAC AACATCAGCC
    CCATTGTTTC GTCTCCAAGT CCGTCGCCAA TGATAACGTC GAGTCTTATG TTGTAGTCGG
         190        200        210        220        230        240

    AACTGGAGTC AGGAT -3'
    TTGACCTCAG TCCTA -5'
         250
```

Sequence VI

```
                5'- CCTGG CTTTACAGGG CCTGCTGTTG CTGCTGGTCA GAAAGTTGGT
                3'- GGACC GAAATGTCCC GGACGACAAC GACGACCAGT CTTTCAACCA
                      260        270        280        290        300

ACTCTCAGCA TTACTGCTAC TGGTCCACAT AACTCAGTAT CTATTGCAGG TAAAGGGGCT
TGAGAGTCGT AATGACGATG ACCAGGTGTA TTGAGTCATA GATAACGTCC ATTTCCCCGA
      310        320        330        340        350        360

TCGGTATCTG GTGGTGTAGC CACTGTCCCG TTCGTTGATG GACAAGGACA GCCTGTTTTC
AGCCATAGAC CACCACATCG GTGACAGGGC AAGCAACTAC CTGTTCCTGT CGGACAAAAG
      370        380        390        400        410        420

CGTGGGCGTA TTCAGGGAGC CAATATTAAT GACCAAGCAA ATACTGGAAT TGACGGGCTT
GCACCCGCAT AAGTCCCTCG GTTATAATTA CTGGTTCGTT TATGACCTTA ACTGCCCGAA
      430        440        450        460        470        480

GCAGGTTGGC GAGTTGCCAG CTCTCAAGAA ACGCTAAATG TCCCTGTCAC AACCTTTGGT
CGTCCAACCG CTCAACGGTC GAGAGTTCTT TGCGATTTAC AGGGACAGTG TTGGAAACCA
      490        500        510        520        530        540

AAATCGACCC TGCCAGCAGG TACTTTCACT GCGACCTTCT ACGTTCAGCA GTATCAAAAC
TTTAGCTGGG ACGGTCGTCC ATGAAAGTGA CGCTGGAAGA TGCAAGTCGT CATAGTTTTG
      550        560        570        580        590        600

TAATTTAATT TAAACTTTAT AAATGCCCTC AATATGAGCG AGTTTGGATA ATTTTATTAT
ATTAAATTAA ATTTGAAATA TTTACGGGAG TTATACTCGC TCAAACCTAT TAAAATAATA
      610        620        630        640        650        660
```

TTTAAAAATA TCTATTTTGA ATAGATAGGT TTTATGCTTC CATGCAAAAA CTTAAAGAGG
AAATTTTTAT AGATAAAACT TATCTATCCA AAATACGAAG GTACGTTTTT GAATTTCTCC
670 680 690 700 710 720

GATTATGTAT ATTTTGAATA AATTTATACG TAGAACTGTT ATCTTTTTCC TTTTTTTTGC
CTAATACATA TAAAACTTAT TTAAATATGC ATCTTGACAA TAGAAAAAGG AAAAAAAACG
730 740 750 760 770 780

TACCTTCCAA TTGCTTCTTC GGAAAGTAAA AAAATTGAGC AACCATTATT AACACAAAAA
ATGGAAGGTT AACGAAGAAG CCTTTCATTT TTTTAACTCG TTGGTAATAA TTGTGTTTTT
790 800 810 820 830 840

TATTATGGCC TAAGATTGGG CACTACACGT GTTATTTATA AAGAAGATGC TCCATCAACA
ATAATACCGG ATTCTAACCC GTGATGTGCA CAATAAATAT TTCTTCTACG AGGTAGTTGT
850 860 870 880 890 900

AGTTTTTGGA TTATGAATGA AAAAGAATAT CCAATCCTTG TTCAAACTCA AGTATATAAT
TCAAAAACCT AATACTTACT TTTTCTTATA GGTTAGGAAC AAGTTTGAGT TCATATATTA
910 920 930 940 950 960

GATGATAAAT CATCAAAAGC TCCATTTATT GTAACACCAC CTATTTTGAA AGTTGAAAGT
CTACTATTTA GTAGTTTTCG AGGTAAATAA CATTGTGGTG GATAAAACTT TCAACTTTCA
970 980 990 1000 1010 1020

AATGCGCGAA CAAGATTGAA GGTAATACCA ACAAGTAATC TATTCAATAA AAATGAGGAG
TTACGCGCTT GTTCTAACTT CCATTATGGT TGTTCATTAG ATAAGTTATT TTTACTCCTC
1030 1040 1050 1060 1070 1080

```
TCTTTGTATT GGTTGTGTGT AAAAGGAGTC CCACCACTAA ATGATAATGA AAGCAATAAT
AGAAACATAA CCAACACACA TTTTCCTCAG GGTGGTGATT TACTATTACT TTCGTTATTA
      1090       1100       1110       1120       1130       1140

AAAAACAACA TAACTACGAA TCTTAATGTG AATGTGGTTA CGAATAGTTG TATTAAATTA
TTTTTGTTGT ATTGATGCTT AGAATTACAC TTACACCAAT GCTTATCAAC ATAATTTAAT
      1150       1160       1170       1180       1190       1200

ATTTATAGGC CTAAAACTAT AGACTTAACG ACAATGGAGA TTGCAGATAA ATTAAAGTTA
TAAATATCCG GATTTTGATA TCTGAATTGC TGTTACCTCT AACGTCTATT TAATTTCAAT
      1210       1220       1220       1240       1250       1260

GAGAGAAAAG GAAATAGTAT AGTTATAAAG AATCCAACAT CATCATATGT GAATATTGCA
CTCTCTTTTC CTTTATCATA TCAATATTTC TTAGGTTGTA GTAGTATACA CTTATAACGT
      1270       1280       1290       1300       1310       1320

AATATTAAAT CTGGTAATTT AAGTTTTAAT ATTCCAAATG GATATATTGA GCCATTTGGA
TTATAATTTA GACCATTAAA TTCAAAATTA TAAGGTTTAC CTATATAACT CGGTAAACCT
      1330       1340       1350       1360       1370       1380

TATGCTCAAT TACCTGGTGG AGTACATAGT AAAATAACTT TGACTATTTT GGATGATAAC
ATACGAGTTA ATGGACCACC TCATGTATCA TTTTATTGAA ACTGATAAAA CCTACTATTG
      1390       1400       1410       1020       1430       1440

GGCGCTGAAA TTATAAGAGA ATTATTAGTT TAAGGTGTAA AACAAATGAA GAAAACCACA
CCGCGACTTT AATATTCTCT TAATAATCAA ATTCCACATT TTGTTTACTT CTTTTGGTGT
      1450       1460       1470       1480       1490       1500
```

ATTACTCTAT TTGTTTTAAC CAGTGTATTT CACTCTGGAA ATGTTTTCTC CAGACAATAT
TAATGAGATA AACAAAATTG GTCACATAAA GTGAGACCTT TACAAAAGAG GTCTGTTATA
1510 1520 1530 1540 1550 1560

AATTTCGACT ATGGAAGTTT GAGTCTTCTC CCGGTGAGAA TGCATCTTTT CTAAGTGTTG
TTAAAGCTGA TACCTTCAAA CTCAGAAGAG GGCCACTCTT ACGTAGAAAA GATTCACAAC
1570 1580 1590 1600 1610 1620

AAACGCTTCC CTGGTAATTA TGTTGTTGAT GTATATTTGA ATAATCAGTT AAAAGAAACT
TTTGCGAAGG GACCATTAAT ACAACAACTA CATATAAACT TATTAGTCAA TTTTCTTTGA
1630 1640 1650 1660 1670 1680

ACTGAGTTGT ATTTCAAATC AATGACTCAG ACTCTAGAAC CATGCTTAAC AAAAGAAAAA
TGACTCAACA TAAAGTTTAG TTACTGAGTC TGAGATCTTG GTACGAATTG TTTTCTTTTT
1690 1700 1710 1720 1730 1740

CTTATAAAGT ATGGGATCGC CATCCAGGAG CTTCATGGGT TGCAGTTTGA TAATGAACAA
GAATATTTCA TACCCTAGCG GTAGGTCCTC GAAGTACCCA ACGTCAAACT ATTACTTGTT
1750 1760 1770 1780 1790 1800

TGCGTTCTCT TAGAGCATTC TCCTCTTTAA ATATACTTAT AACGCGGCTA ACCAAAGTTT
ACGCAAGAGA ATCTCGTAAG AGGAGAAATT TATATGAATA TTGCGCCGAT TGGTTTCAAA
1810 1820 1830 1840 1850 1860

GCTTTTAAAT GCACCATCTA AAATTCTATC TCCAATAGAC AGTGAAATTG CTGATGAAAA
CGAAAATTTA CGTGGTAGAT TTTAAGATAG AGGTTATCTG TCACTTTAAC GACTACTTTT
1870 1880 1890 1900 1910 1920

```
TATCTGGGAT GATGGCATTA ACGCTTTTCT TTTAAATTAC AGAGCTTAAT TATTTGCATT
ATAGACCCTA CTACCGTAAT TGCGAAAAGA AAATTTAATG TCTCGAATTA ATAAACGTAA
      1930       1940       1950       1960       1970       1980

CTAAGGTTGG AGGAGAGAGA TTCATACTTT GGTCAAATTC AACCTTGGTT TTAATTTTGG
GATTCCAACC TCCTCTCTCT AAGTATGAAA CCAGTTTAAG TTGGAACCAA AATTAAAACC
      1990       2000       2010       2020       2030       2040

TCCCTGGCGG CTAAGGAATC TATCATCTTG GCAAAACTTG TCAAGCGAAA AAAAATTTGA
AGGGACCGCC GATTCCTTAG ATAGTAGAAC AGTTTTGAAC AGTTCGCTTT TTTTTAAACT
      2050       2060       2070       2080       2090       2100

ATCAGCATAT ATTTATGCTG AGCGAGGTTT AAAAAAAATA AAGAGCAAAC TAACAGTTGG
TAGTCGTATA TAAATACGAC TCGCTCCAAA TTTTTTTTAT TTCTCGTTTG ATTGTCAACC
      2110       2120       2130       2140       2150       2160

GGACAAATAT ACCAGTGCAG ATTTATTCGA TAGCGTACCA TTTAGAGGCT TTTCTTTAAA
CCTGTTTATA TGGTCACGTC TAAATAAGCT ATCGCATGGT AAATCTCCGA AAAGAAATTT
      2170       2180       2190       2200       2210       2220

TAAAGATGAA AGTATGATAC CTTTCTCACA GAGAACATAT TATCCAACAA TACGTGGTAT
ATTTCTACTT TCATACTATG GAAAGAGTGT CTCTTGTATA ATAGGTTGTT ATGCACCATA
      2230       2240       2250       2260       2270       2280

TGCGAAAACC AATGCGACTG TAGAAGTAAG ACAAAATGGA TACTTGATAT ATTCTACTTC
ACGCTTTTGG TTACGCTGAC ATCTTCATTC TGTTTTACCT ATGAACTATA TAAGATGAAG
      2290       2300       2310       2320       2330       2340
```

AGTCCCCCCC GGGCAATTCG AGATAGGTAG AGAACAAATT GCTGATC -3'
TCAGGGGGGG CCCGTTAAGC TCTATCCATC TCTTGTTTAA CGACTAG -5'
2350 2360 2370 2380

or sequences degeneratively equivalent thereto.

6. Recombinant DNA as claimed in Claim 2 wherein the sequences I and II are comprised within a contiguous sequence VII (as described herein).

7. Recombinant DNA as claimed in Claim 3 wherein the sequences III and IV are comprised within a contiguous sequence VIII (as described herein).

8. Recombinant DNA as claimed in Claim 5 wherein the sequences V and VI are comprised within a contiguous sequence IX (as described herein).

9. Recombinant DNA as claimed in Claim 3 or Claim 7 wherein the amino acid sequence encoded is all or part of an allele of SEFA.

10. Recombinant DNA as claimed in any one of claims 1 to 5 further comprising a sequence encoding for a further amino acid sequence.

11. Recombinant DNA as claimed in Claim 10 wherein the further amino acid sequence comprises additional SEFA antigen or epitopic parts thereof.

12. Recombinant DNA as claimed in Claim 10 wherein the further amino acid sequence comprises a non-SEFA epitopic sequence.

13. Recombinant DNA as claimed in Claim 12 wherein the non-SEFA epitopic sequence comprises SB10 epitope of Mycobacterium bovis.

14. A novel plasmid comprising recombinant DNA as claimed in any one of Claims 1 to 13.

15. A plasmid as claimed in Claim 14 comprising a plasmid suitable for transformation of E.coli or yeast into which the recombinant DNA has been inserted.

16. A plasmid as claimed in Claim 14 or 15 comprising pBR322, pACYC184 or pUC18 into which the recombinant DNA has been inserted.

17. A transformant microorganism comprising a plasmid as claimed in any one of claims 14, 15 or 16.

18. A microorganism as claimed in Claim 17 wherein the plasmid host is a yeast or an E.coli.

19. A microorganism as claimed in Claim 18 wherein the plasmid host is an E. coli DH5alpha.

20. A plasmid as claimed in any one of Claims 14, 15 or 16 wherein the recombinant DNA sequences are produced by extracting total genomic DNA from an S. enteritidis or a SEFA expressing S. dublin; partially digesting the genomic DNA with SauIIIA restriction endonuclease to provide fragments in the size range 5 to 10 kilobases; ligating the fragments into a plasmid pBR322, pACYC184 or pUC18 and selecting desired plasmids for their ability to express SEFA, a part thereof or an allele of either.

21. A plasmid as claimed in Claim 20 wherein a further DNA sequence has been ligated into the BamH1 site in sequence I, III, V, VII, VIII or IX.

22. A plasmid as claimed in Claim 20 wherein the further DNA sequence is in frame with the SEFA expressing sequence.

23. A transformant microrganism as claimed in any one of Claims 17 to 19 wherein the plasmid is that as claimed in any one of Claims 20 to 22.

24. A polypeptide or oligopeptide comprising SEFA, an epitopic part thereof or alleles of either as expressed by a transformant as claimed in any one of Claims 17, 18, 19 or 23.

25. A test kit for the identification of microorganisms as being of either serotype S. enteritidis or S. dublin comprising a polypeptide as claimed in Claim 24.

26. A method for the determination of the presence of microorganisms having DNA or RNA polynucleotide sequence encoding for SEFA, an epitopic part thereof or alleles of either, or such DNA or RNA itself, comprising:

(a) providing a sample suspected of containing said encoding polynucleotide sequence;

(b) determining the presence of said sequence by monitoring hybridization of SEFA sequence targeted polynucleotide hybridization probes with said DNA or RNA.

27. A method as claimed in Claim 26 wherein the polynucleotide probes are targeted to any one of the sequences VII, VIII or XI.

28. A method as claimed in Claim 27 wherein the polynucleotide probe consists of sequence VII, VIII or XI.

29. A method for determining the presence of microorganisms having DNA or RNA polynucleotide sequence encoding for SEFA or an epitopic part thereof, or such DNA or RNA itself, comprising:

(a) providing a sample suspected of containing said encoding polynucleotide sequence;

(b) subjecting said sample to conditions under which polynucleotide sequences comprising sequences (I) and (II) are replicated by use of the polymerase chain reaction;

(c) determining the presence of any sequence produced.

30. A method as claimed in Claim 29 wherein the step (c) is carried out using a polynucleotide hybridization probe.

31. A method as claimed in either of Claim 29 or Claim 30 wherein step (b) employs primer pairs comprising one primer selected from group (A) and the other from group (B):

Group A:	Group B:
5' -GTGCGAATGCTAATAGTTGA- 3'	5' -AAAACAGGCTGTCCTTGTCCA- 3'
5' -TGCGTAAATCAGCATCTGCA- 3'	5' -TTAGCGTTTCTTGAGAGCTGG- 3'
5' -TCTGCAGTAGCAGTTCTTGC- 3'	5' -TTTTGATACTGCTGAACGTAG- 3'
5' -GCTCAGAATACAACATCAGCCAA- 3'	

32. A method as claimed in any one of Claims 29 to 31 wherein the step (c) is carried out using an oligonucleotide probe selected from sequences of either of groups A or B (as described herein) which is different to that of either of the primers used for step (b).

33. A test kit for performing the method of any one of Claims 26 to 28 comprising polynucleotide hybridization probes targeted at sequence VII, VIII.

34. A test kit as claimed in Claim 33 wherein the probes comprise sequences comprising sequence VII or VIII.

35. A test kit for performing the method of any one of Claims 29 to 32 comprising primers and probes having sequences selected from the groups (A) and (B).

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01691

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/31; C12N15/62; C12Q1/68; G01N33/569
C07H21/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12Q ; G01N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	JOURNAL OF BACTERIOLOGY vol. 170, no. 9, September 1988, AMERICAN SOCIETY FOR MICROBIOLOGY pages 4216 - 4222; FEUTRIER, J. ET AL.: 'Cloning and expression of a Salmonella enteritidis fimbrin gene in Escherichia coli' see the whole document	1-9, 14-15, 17-18, 24,26-27
X	JOURNAL OF BACTERIOLOGY vol. 168, no. 1, October 1986, AMERICAN SOCIETY FOR MICROBIOLOGY pages 221 - 227; FEUTRIER, J. ET AL.: 'Purification and characterization of fimbriae from Salmonella enteritidis'	1,9,11, 24,26-27
Y A	see the whole document	10,12-23 2-8,25, 28-35

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 JANUARY 1992	16 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 910 967 (PRAXIS BIOLOGICS, INC.) 16 November 1989 see the whole document	10,12-23
A	JOURNAL OF GENERAL MICROBIOLOGY vol. 136, no. 2, February 1990, COLCHESTER, GB pages 265 - 272; RADFORD, A.J. ET AL.: 'Epitope mapping of the Mycobacterium bovis secretory protein MPB70 using overlapping peptide analysis' cited in the application see the whole document	10,12-23
A	EP,A,0 383 509 (ORTHO DIAGNOSTIC SYSTEMS, INC.) 22 August 1990 see the whole document	26-35

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101691 SA 51809

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/01/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8910967	16-11-89	AU-A- 3697989	29-11-89
		EP-A- 0419513	03-04-91
EP-A-0383509	22-08-90	CA-A- 2009708	13-08-90
		JP-A- 2295498	06-12-90

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/31, C07K 14/255, G01N 33/50 — A1

(11) International Publication Number: WO 98/03656

(43) International Publication Date: 29 January 1998 (29.01.98)

(21) International Application Number: PCT/US97/12639

(22) International Filing Date: 18 July 1997 (18.07.97)

(30) Priority Data:
60/022,191 19 July 1996 (19.07.96) US

(71) Applicant *(for all designated States except US)*: REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; Morrill Hall, 100 Church Street, S.E., Minneapolis, MN 55455 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: RAJASHEKARA, Gireesh [IN/US]; Minn. Graduate Club, 2089 Carter Avenue, St. Paul, MN 55108 (US). NAGARAJA, Kakambi, V. [IN/US]; 2235 Milton Street North, Roseville, MN 55113 (US). KAPUR, Vivek [IN/US]; Apartment 108, 2572 Kenzie Terrace, St. Anthony, MN 55418 (US).

(74) Agent: BRUESS, Steven, C.; Merchant, Gould, Smith, Edell, Welter & Schmidt, P.A., 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402-4131 (US).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECOMBINANT SEF14 FIMBRIAL PROTEIN FROM SALMONELLA

(57) Abstract

A truncated SE fimbria antigen useful as an antigen for immunoassay diagnosis of *Salmonella enteritidis* (SE) infection or evidence of infection.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

RECOMBINANT SEF14 FIMBRIAL PROTEIN FROM SALMONELLA

Field of the Invention

The present invention relates to a method of cloning and expressing a truncated form of a fimbrial gene and the use of the truncated fimbrial gene product in an immunodiagnostic assay and for immunoprophylaxis.

Background of the Invention

Foodborne infections cause an estimated 6.5 million cases of human illness and 9000 deaths annually in the United States alone. Bacterial infections by *Salmonella* are the most commonly reported cause of foodborne outbreaks. *Salmonella enteritidis* (SE) is the dominant *Salmonella* serotype isolated from cases of food poisoning. Many of these outbreaks are thought to be due to infected poultry products, particularly eggs and egg products.

The best way to prevent infection in human populations is to diagnose and treat the infected animal prior to human consumption. Because the greatest threat of food poisoning from *Salmonella* is from poultry products, there is a need for a method to detect birds that are infected with SE.

Some current diagnostic methods rely on conventional bacteriologic cultures. However, these procedures are relatively slow, often taking up to 3 to 4 days to provide even a presumptive diagnosis. Additionally, the great susceptibility of SE to physical and chemical factors such as desiccation, radiation, low temperature, heating, or chemical preservatives, causes traditional bacteriologic culture methods to generally have a low sensitivity. Consequently, many birds or animals

that are infected with SE are often not detected when conventional bacterial cultures are used.

Other diagnostic methods rely on the detection of serum antibodies specific to SE. Although several serological methods such as micro-agglutination, serum plate agglutination, latex particle agglutination microantiglobulin, ELISA have previously been employed, these assays lack either the sensitivity or specificity necessary to detect SE infected birds, or the tests are too difficult to perform in a routine laboratory or field setting. Consequently, widespread application of these tests for the detection of SE infections has been impractical.

A useful antigenic determinant that is found on many species of *Enterobacteriaceae* are fimbriae, proteinaceous filamentous surface structures composed of protein subunits called fimbrin. Upon infection, birds make antibodies to this SE fimbrial antigen. Therefore, the SE fimbrial antigen is useful in a diagnostic assay for the presence of SE in poultry.

SE is known to have at least four distinct fimbria, designated Sef14, Sef17, Sef18 and Sef21. These proteins are encoded by *SefA*, *AgfA*, *SefD* and *FinA genes*, respectively.

Although the gene encoding Sef14 has been identified and its DNA nucleotide sequence determined (Trucotte and Woodward, *Journal of General Microbiology*, 139:1477-1485 (1993)), an effective diagnostic method using this surface antigen has not been developed, partially due to the difficulty of efficiently producing the fimbriae proteins in purified form and in large quantities. Additionally, expression of Sef14 fimbriae by cultured

Salmonella enteritidis is highly dependent on the growth medium composition. In a study by Thorns et al., *International Journal of Food Microbiology*, 21:47-53 (1994), only peptone water pH 7.2 supported the expression of Sef14 by all *Salmonella enteritidis* strains examined. Consequently, previous diagnostic assays using Sef14 have used antibodies against Sef14 and not the antigen itself.

Hence, there is a need for a sensitive, specific and routine antigen and method to reliably detect SE infection in birds, preferably a method that is easily adaptable to large-scale screening of poultry flocks.

Summary of the Invention

The present invention provides a sensitive, specific, routine antigen and assay to reliably detect SE-infected animals. Specifically, the present invention provides a truncated form of the Sef14 antigen that can be easily produced in purified form and in large quantities and used in the method of the invention. The novel Sef14 antigen, when coupled to a substrate such as latex beads, provides a diagnostic assay for SE, particularly useful in large-scale screening of poultry flocks.

Brief Description of the Figures

Figure 1 is a photograph showing a SDS-PAGE of the recombinant Sef14 (rSef14) fragment (arrow).

Figure 2 is a photograph showing a Western blot of the rSef14 fragment probed with anti-Sef14 antibody (lane 1) and anti-tag (T7) antibody (lane 2).

Figure 3 is a photograph showing results of a rSef14-latex agglutination assay for SE infection in

chickens exposed to *S. enteritidis* (A), *S. pullorum* (B), and serum-free antigen control (C).

Figure 4 is a photograph showing results of a rSef14-latex agglutination assay for SE infection in chickens exposed to *S. enteritidis* (A), *S. gallinarum* (B), *S. pullorum* (C), *S. typhimurium* (D), *C. arizonae* (E), *E. coli* (F), serum free antigen control (G), and serum control (H).

Figure 5 is a graph showing the percentage of chickens testing positive for anti-SE antibodies during 4 weeks post-innoculation. The five bars at each week represent innoculation with 10^4, 10^6, 10^8, 10^{10}, and control (no cells).

Figure 6 is a graph showing the antibody titres of chicken sera samples testing positive for anti-SE antibodies.

Figure 7 is a graph showing the antibody titres of chicken egg yolk samples testing positive for anti-SE antibodies.

Detailed Description of the Invention

The present invention is directed to a method for diagnosing *Salmonella enteritidis* infection or evidence of infection in an animal, particularly poultry, using a recombinant truncated fimbrial antigen.

"Infection" means active colonization of the animal by SE organisms. "Evidence of infection" means a prior history of colonization by SE in the animal, although tive colonization is not present. Diagnosis of active ction is needed to protect against contamination of upplies, whereas diagnosis of prior infection is

needed to alert against new infection or to trace the source of infection in a flock.

Fimbrial Proteins

Fimbriae are proteinaceous filamentous surface structures composed of protein subunits called fimbrin. These proteinaceous structures are thought to be virulence factors which mediate specific attachment to host cell mucosal surfaces. They are present in most enteric bacteria capable of invading host cells.

Salmonella enteritidis has four distinct fimbriae: Sef14, Sef17, Sef18 and Sef21 which are encoded by *sefA, agfA, sefD* and *fimA* genes, respectively. Sef14 is unique with only limited distribution in the genus. In contrast, all other fimbrial proteins are widely distributed in the genus. Thus, they have limited use as diagnostic reagents for SE detection.

Cloning and Expression of Sef14

In the present invention, a truncated form of the Sef14 antigen retaining the antigenic character of the entire protein has been produced. Unlike the complete protein, however, the truncated form can be easily produced in purified form and in large quantities, without special growth medium requirements.

PCR technology is used to produce the truncated Sef14 protein by amplification with suitable primers.

Primers are selected to amplify the gene encoding Sef14 in a region downstream of the encoded signal peptide, e.g., downstream of about nucleotide 145 of the DraI genomic fragment shown in Figure 1 of Turcotte and Woodward, *Supra*. Preferably, the PCR primers include

additional nucleotides at the 5' ends, encoding specific restriction enzyme recognition sequences, for ease of purification. For example, useful primers for amplifying that portion of the *sefA* gene encoding an immunogenic Sef14 fragment downstream of the signal peptide are shown below:

GGGAATTCGCTGGCTTTGTTGGTAACA	SEQ ID NO:1
GGGCTCGAGTTAGTTTTGATACTGAACGTA	SEQ ID NO:2

After a truncated gene sequence encoding Sef14 is produced, it can be cloned into a host using a plasmid or phage as a vector. Typically, the expression of Sef14 fimbriae by cultured *Salmonella enteritidis* is highly dependent on the growth medium composition (Thorns et al, *International Journal of Food Microbiology*, 21:47-53 (1994)), and it is typically difficult to produce large quantities. However, a truncated form of Sef14 having at least the signal peptide removed is expressed in host systems such as *E. coli* without these difficulties.

Truncated Sef14 Antigen

Because the truncated Sef14 protein retains the antigenic characteristics of the complete protein, it is useful in various immunological methods. For example, the inventive antigen is useful in antibody binding immunoassays such as assays to detect the presence of antibodies against SE in a sample. Suitable binding assays include ELISA, wherein the recombinant Sef14 antigen is bound to a surface and exposed to antibodies against SE. To detect the presence of bound anti-SE antibodies, a marker such as an enzyme-linked secondary antibody is then added.

An agglutination assay using truncated Sef14 antigen-coated latex beads is preferred. In the agglutination reaction, antigen-coated latex beads form detectable clusters when exposed to antibodies against SE. This preferred assay is described more fully in Example 4, below.

Diagnostic Assays

The assays described above can be used to detect the presence of antibodies to *Salmonella enteritidis*. Preferably, the assays are used to determine whether or not an animal, e.g. a poultry animal such as a chicken or turkey, is infected with SE. Animal fluid such as blood or serum can be used in a diagnostic assay. If an animal is infected with SE, the animal will typically produce anti-SE antibodies. The recombinant Sef14 antigen is used to detect the presence of anti-SE antibodies, SE infection or the SE organism itself. Diagnostic assays such as these are particularly useful in birds. More particularly, diagnostic assays are useful in detecting SE infections in chicken or turkey to prevent foodborne illness by poultry consumption.

Vaccine

Passive immunization with anti-Sef14 antibodies has been shown to reduce *Salmonella enteritidis* colonization (Peralta et al. 1994). Additionally, Sef14 can induce a T-cell immune response (Ogunniyi et al 1994). Because the truncated Sef14 antigen exhibits these immunological activities, can be produced in large quantities, and does not have the cumbersome growth requirements of the complete protein, the truncated Sef14

antigen is also useful as a vaccine to confer immunity against SE. Preferably, the truncated Sef14 antigen is used as a vaccine in poultry to prevent foodborne illnesses.

EXAMPLES

The invention may be better understood with reference to the following examples which are not intended to limit the invention.

Example 1

Isolation of *S. enteritidis* genomic DNA

S. enteritidis was grown overnight at 37°C in Luria-Bertani (LB) broth. Genomic DNA was extracted as described (Sambrook, et al., 1989) using standard methods with minor modifications. In brief, bacterial cells were pelleted by centrifugation at 13,000 x g for 3 minutes, washed/suspended in 1 ml of 1 M NaCl, centrifuged for 5 minutes at 13,000 x g, and the pellet resuspended in 1 ml TE buffer (50 mM Tris-HCl, 50 mM EDTA, pH 7.8). The sample was next incubated with 5 µl of lysozyme (50 mg/ml) (Sigma Chemical Co., St. Louis, MO) and 0.3 mg/ml RNase A (Sigma) at 37°C for 30 minutes. To this suspension, 1% sarkosyl and 0.6 mg/ml of proteinase K (Sigma) were added, and the mixture incubated at 37° for 1 hour. Following incubation, chromosomal DNA was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). Genomic DNA in the aqueous phase was precipitated at -20°C with two volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate, and

pelleted by centrifugation at 13,000 x g for 5 minutes. The pellet was then washed twice with 70% ethanol, air dried, and suspended in TE Buffer (10mM Tris-HCl and 1mM EDTA pH 8.0). Total DNA was quantitated spectrophotometrically at A_{260} nm.

Example 2

Cloning of *sefA* gene fragment

Oligonucleotide primer selection and synthesis:

Oligonucleotide primers corresponding to an internal fragment (64-498 bp) of the open-reading frame of the *sefA* gene were used for PCR amplification. Additional bases were added to the 5' end of each primer in order to confer a recognition sequence for either *EcoRI* (forward primer) or *XhoI* (reverse primer). The oligonucleotide primers were obtained from Integrated DNA technologies Inc., Ames, IA. The DNA sequences for the forward and reverse primers are shown below:

GGGAATTCGCTGGCTTTGTTGGTAACA	SEQ ID NO:1
GGGCTCGAGTTAGTTTTGATACTGAACGTA	SEQ ID NO:2

Additional nucleotides added to the 5' end of the primers are underlined.

PCR amplification of *sefA* gene fragment.

Amplification reactions were performed in 30 µl volumes with 30 pmol of each primer and 5 mM $MgCl_2$. The reagents and enzymes used for PCR were obtained either from Boehringer Mannheim (Indianapolis, IN) or Perkin Elmer (Foster City, CA). One hundred ng of genomic DNA was used as a template for PCR amplification with the following parameters: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C for 1.5

minutes), annealing 52°C for 1 minute) and extension (72°C for 2 minutes), and a final extension of 15 minutes at 72°C. All amplification reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler (Model 480). The PCR products were analyzed on a 1% agarose gel, stained with ethidium bromide (0.5µg/ml), and photographed under UV light.

PCR products were gel extracted (Qiagen Inc., Chatsworth, CA), quantitated spectophometrically, at 260 nm, and cloned directly into pGEM-T vector (Promega, Madison, WI). Following ligation, 2µl of the reaction products were transformed into *E. coli* DH5α cells (Gibco BRL, Gaithersburg, MD) by the heat shock method. Recombinant colonies were selected on ampicillin/IPTG-Xgal containing plates and screened for the presence of the appropriate insert by restriction analysis.

Nucleotide sequence analysis

A bacterial colony containing the recombinant plasmid with the *rSefA* fragment was grown in LB-ampicillin media, and the plasmid extracted using Qiagen plasmid extraction kit (Qiagen). The nucleotide sequence of the insert was determined using oligonucleotide primers specific to the vector sequence by automated DNA sequencing at the University of Minnesota Advanced Genetic Analysis Center. The insert was sequenced in its entirety in both orientations, and the amino acid sequence deduced using the standard genetic code (DNA*, Madison, WI). Sequencing results are shown below for nucleotide and deduced amino acid sequences of the insert (Seq.ID.NO:5), together with a tag sequence added during the subcloning of the fragment into the pET/abc expression vector (Seq.ID.NO:3). The

added tag sequence at the 5' end, provides a Histidine-rich portion to facilitate purification of the sequence on nickel columns, as well as an antigenic region that specifically binds the T7 anti-tag antibody provided with the pET/abc vector kit.

Nucleic Acid Sequence encoding rSefA fragment

SEQ ID NO:3

```
ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG  45
CCG CGC GGC AGC CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG  90
GGT CGC GGA TGG GAA TTC GCT GGC TTT GTT GGT AAC AAA GCA GTG  135
GTT CAG GCA GCG GTT ACT ATT GCA GCT CAG AAT ACA ACA TCA GCC  180
AAC TGG AGT CAG GAT CCT GGC TTT ACA GGG CCT GCT GTT GCT GCT  225
GGT CAG AAA GTT GGT ACT CTC AGC ATT ACT GCT ACT GGT CCA CAT  270
AAC TCA GTA TCT ATT GCA GGT AAA GGG GCT TCG GTA TCT GGT GGT  315
GTA GCC ACT GTC CCG TTC GTT GAT GGA CAA GGA CAG CCT GTT TTC  360
CGT GGG CGT ATT CAG GGA GCC AAT ATT AAT GAC CAA GCA AAT ACT  405
GGA ATT GAC GGG CTT GCA GGT TGG CGA GTT GCC AGC TCT CAA GAA  450
ACG CTA AAT GTC CCT GTC ACA ACC TTT GGT AAA TCG ACC CTG CCA  495
GCA GGT ACT TTC ACT GCG ACC TTC TAC GTT CAG CAG TAT CAA AAC  540
TAA CTC GAG CCC  552
```

*Additional amino acid residues added to the amino terminus to facilitate protein purification and cloning are underlined.

Deduced amino acid sequence of *rSefA* protein fragment*

Seq. ID NO: 4

```
MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSEFAGFVGNKAVVQAAVT
IAAQNTTSANWSQDPGFTGPAVAAGQKVGTLSITATGPHNSVSIAGKGASVSGG
VATVPFVDGQGQPVFRGRIQGANINDQANTGIDGLAGWRVASSQETLNVPVTT
FGKSTLPAGTFTATFYVQQYQN
```

*Additional amino acid residues added to the amino terminus to facilitate protein purification and cloning are underlined.

It is understood that the amino acids added to the N-terminus of the Spf14 antigen are optional, and used for ease of cloning and purification. The amino acid sequence in the absence of these added residues (Sequence ID No:6)

with or without other added residues for cloning or purification procedures, for example, are similarly useful as antigens in the diagnostic assays of the invention.

Subcloning *sefA* gene fragment into an expression vector

The pGEM-T plasmid carrying *sefA* fragment was double digested with *EcoRI* and *XhoI*, and the digested products gel purified (Qiagen) and cloned into *EcoRI* and *XhoI* digested pET/abc expression vectors (Novagen Inc., Madison, WI). Ligation products (2 µl) from each of the reactions were transformed into *E. coli* BL21(DE3)pLyS cells by heat shock method. Recombinant clones were cultured on kanamycin and chloramphenicol containing plates, and analyzed by restriction enzyme analysis.

rSefA fragment expression

The recombinant clones were selected based on restriction enzyme analysis with *EcoRI* and *XhoI* digestion, selecting those clones yielding appropriately sized fragments as compared with a vector control. Selected clones were analyzed for *rSefA* fragment expression. Briefly, a single colony from each (pETabc/*SefA* fragment) freshly streaked plate was picked and inoculated to 50 ml LB broth containing appropriate antibiotics and incubated with shaking at 200 rpm at 37°C until the OD_{600} reached 0.6. Cultures were induced with IPTG (0.4 mM) and incubated for an additional 3 hours. Following incubation, the cells were pelleted and resuspended in 5 ml of TE buffer (50mM Tris-HCl pH 8.0, 2mM EDTA) and incubated with 25 µl of lysozyme (50 mg/ml) and 100 µl of 1% Triton X-100 for 20 minutes at 30°C. The samples were sonicated until they were no longer viscous, and centrifuged at 39,000 x g for

20 minutes. The supernatant was passed through a 0.45 µm membrane filter, and stored at -20°C until further use.

SDS-PAGE analysis

The cell lysates were next analyzed by SDS-PAGE for the presence of the *rSefA* fragment by mixing with an equal volume of 2x SDS solubilization buffer separating on 12% polyacrylamide gels, and staining with Coomassie blue. The results are shown in lane 1 of Figure 1 which contains the total protein produced by the vector and contained in the cell lysates.

Western blot analysis

The lysates were separated on 12% polyacrylamide gels and transferred onto a nitrocellulose membrane using Transblot apparatus (Bio-Rad laboratories, Hercules, CA). Following transfer, the membrane was blocked with 3% BSA in phosphate buffered saline (PBS) and stained with either T7 anti-tag antibody (Novagen) or rabbit anti-Sef14 specific antibody (kindly provided by Dr. W. W. Kay, University of Victoria, BC, Canada). The membrane was washed and stained with anti-rabbit IgG/HRP conjugate and treated with developing reagent (Amersham lif sciences, Inc., USA) for 1 minute, exposed to X-ray film, and the radiograph developed. The results are shown in Figure 2, where lane 1 is probed with anti-Sef14 antibody, and lane 2 with T7 anti-tag antibody.

Purification of rSef14 fragment protein by column chromatography and electroelution

The recombinant Sef14 protein fragment produced in the cell lysates described above was purified by binding

of the Histidine-rich tag to nickel columns as described by the manufacturer (Novagen). Briefly, the cells were induced and extract was prepared as described above except that the induced cells were suspended in Tris buffer without EDTA. The cell lysate was passed through nickel columns and washed sequentially with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-CHl, pH 7.9) and wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). The bound protein was eluted using elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), quantitated using a Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA), and analyzed by SDS-PAGE. (See Figure 1, lane 2, where the arrow indicates the rSef14 fragment at about 19 KDa.) Since the column purified recombinant material contained traces of non-specific proteins, the appropriate rSef14 fragment was further purified by cutting the rSef14 fragment from the gel and electroelution (Bio-Rad) following the manufacturer's suggested protocol. The electroluted fragment is shown in lane 4 of Figure 1 (at arrow).

Example 3

Covalent coupling of rSef14 to blue-dyed latex beads

The electroeluted rSef14 protein fragment was coupled to either 0.5 µm or 1.0 µm blue-dyed latex beads (Polysciences Inc., Warrington, PA) by gluteraldehyde method. Briefly, 1 ml of 2.5% suspension of the beads were washed with PBS (pH 7.4), pelleted by centrifugation and resuspended in 1 ml of 8% gluteraldehyde (EM grade) in PBS, and incubated overnight with gentle end-to-end mixing at room temperature. Following gluteraldehyde treatment, the beads were pelleted, washed with PBS three times and

incubated with 500 µg of purified rSef14 fragment for 5 hours at room temperature with gentle end-to-end mixing. The beads were pelleted, and incubated with 1 ml of 0.5 M ethanolamine in PBS for 30 minutes at room temperature with gentle end-to-end mixing. The mixture was then treated with 1 ml of 10 mg/ml BSA in PBS for 30 minutes at room temperature, centrifuged and the pellet resuspended in 1 ml PBS (pH 7.4), containing 10 mg/ml BSA, 0.1% NaN_3 and 5% glycerol, and stored at 4°C to form rSef14 - fragment coated latex beads for use in agglutination assays.

Example 4

rSef14-latex bead agglutination test

Bacteria was administered to chickens by either injection, intratracheal or oral administration of 10^7 colony forming units (CFU) of either *S. enteritidis*, *S. pullorum*, *S. arizonae*, *S. typhimurium*, *S. gallinarum*, or *E. coli*. After about two to three weeks exposure, serum was collected and used to evaluate the sensitivity and specificity of the rSef14-latex beads in an agglutination assay for anti-SE antibody binding. A total volume of 7.5 µl of rSef14 fragment coated latex beads, produced as described for Example 3, were mixed with an equal volume of chicken serum collected from birds exposed to various pathogens, as described above. The presence of agglutination, visually seen as a loss of intense blue color in the sample (i.e., lightening of color as the coated beads agglutinate or form a lattice). Absence of the agglutination reaction was visualized by the remaining intense blue color of the dyed beads in a homogeneous suspension. Positive or negative agglutination reaction

was recorded after two minutes. The results are shown in Figures 3 and 4.

In figure 3, intense blue color (negative result) is seen in test samples B and C (*S. pullorum* and the serum-free antigen control). In contrast, a positive agglutination result is seen in test Sample A, (*S. enteritidis*), as a pale blue, diffuse agglutination pattern.

In figure 4, a positive agglutination reaction is seen in sample A (*S. enteritidis*) and in sample H (serum control). No agglutination reaction is seen in the samples B-G containing serum animals exposed to the following pathogens: *S. gallinarium* (B), *S. pullorum* (C), *S. typhimurium* (D), *S. arizonae* (E), *E. coli* (F), and serum free antigen control (H).

Example 5

Detection of anti-S.E. anitbodies in infected chickens

To confirm the specificity of the assay of the invention, forty SPF chickens (age 4weeks) were innoculated with various species of *Salmonella*. A suspension of 10^9 CFU in PBS was administered by injection. A booster dose of 10^9 CFU was administered orally two weeks later. Serum samples were taken at weekly intervals and assayed for the presence of anti-SE antibodies.

Serum samples were assayed by the standard Serum Plate Test (SPT) as described in Schaffer and MacDonald, 1931, _____,___:236-240. Slides were visually scored (+ or -) for the presence of agglutination, indicating antigen-antibody binding. Samples were also assayed by the Microtiter Plate Test (MPT) as described in Williams and Whittemore, 1971 *Applied Microbiology* 21:394-399. These

two assays are standard screening methods for the detection of Salmonella, using *S.pullorium* as a whole-cell antigen, and are not specific for SE, as shown in the table below.

To demonstrate the specificity of the assays of the invention, serum samples were assayed using the latex agglutination test (LAT) described above for Example 4, which utilized the truncated Spf14 antigen coupled to latex beads. Serum samples were also assayed for anti-SE antibodies by ELISA. In the ELISA, the truncated Spf14 antigen prepared as described for Example 3, was coated onto polystyrine plates. Antigen-coated plates were exposed to serum samples to permit binding of anti-SE antibodies to the antigen. The bound antigen-antibody complexes were washed, and then incubated with anti-chicken antibody coupled to biotin. The complex was then exposed to strep-avidin for signal detection.

Results are shown in the table below. The LAT and Elisa assays demonstrated a useful specificity for the detection of SE. Of the organisms tested, only *S.dublin*, a bovine pathogen, demonstrated cross-reactivity in the assays.

Species	SPT	MT	LAT	ELISA
S. enteritidis	+	+	+	+
S. gallinarum	+	+	-	-
S. pullorum	+	+	-	-
S. dublin	+	+	+	+
S. berta	+	+	-	-
S. typhimurium	-	+	-	-
E. coli	-	-	-	-
Control (no cells)	-	-	-	-

Example 6

Specificity of anti-SE assay

The ELISA assay for detecting anti-SE antibodies described above for Example 6 was tested for specificity using a panel of antisera against known pathogenic organisms. Each sera was assayed in the anti-SE ELISA. No crossreativity was observed with any of the tested antisera.

Antisera	ELISA		Antisera	ELISA
Pox	-		MG	-
Reo	-		NDV	-
Rev	-		CAV	-
SB-1	-		HVT	-
IBDV	-		IBV	-
ILT	-		*S.typhimurum*	-
LLA	-		*S.gallinarum*	-
LLB	-		*S.pullorum*	-
MS	-			

Example 7

Sensitivity of ELISA for detection of SE

Fifty white leghorn layer chickens (5 weeks old) were orally innoculated in a single exposure with varied amounts of SE, from 10^4 to 10^{10} CFU in PBS. Serum samples were collected at weekly intervals for up to seven weeks. Eggs were collected for egg yolk antibody detection.

Samples were analized for detection of anti-SE

antibodies using the ELISA described above for Example 6. As shown in Figure 5, control chickens showed no positive reaction in the ELISA assay. Approximately 40-80% of chickens exposed to 10^4, 10^6, 10^8, and 10^{10} CFU of SE tested positive for anti-SE antibodies during the first four weeks post-innoculation. From 4-7 weeks post-innoculation, the data stabilized at about 45% positive detection of anti-SE antibodies.

Antibody titers in the sera and egg yolks of chickens exposed to 10^4, 10^6, and 10^8 CFU of SE and testing positive in the ELISA for anti-SE antibodies are shown in Figures 6 and 7.

These data demonstrate specific detection of anti-SE antibodies using recombinant Sef14-antigen coated latex beads in an agglutination assay and using the antigen as a capture agent in an ELISA. These assays provide a sensitive and specific diagnostic tool for the detection of anti-SE antibodies in animals and for the diagnosis of SE infection.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: REGENTS OF THE UNIVERSITY OF MINNESOTA

(ii) TITLE OF THE INVENTION:
RECOMBINANT FIMBRIAL PROTEIN

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merchant, Gould, Smith, Edell, Welter & Schmidt
(B) STREET: 3100 Norwest Center, 90 South Seventh St
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 18-JUL-1997
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/022,191
(B) FILING DATE: 19-JUL-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kettelberger, Denise M
(B) REGISTRATION NUMBER: 33,924
(C) REFERENCE/DOCKET NUMBER: 600.335WO01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612/371-5268
(B) TELEFAX: 612/332-9081
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAATTCGC TGGCTTTGTT GGTAACA 27

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGCTCGAGT TAGTTTTGAT ACTGAACGTA 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 552 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...540
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG      48
Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro
 1               5                  10                  15

CGC GGC AGC CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGC      96
Arg Gly Ser His Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg
            20                  25                  30
```

```
GGA TGG GAA TTC GCT GGC TTT GTT GGT AAC AAA GCA GTG GTT CAG GCA      144
Gly Trp Glu Phe Ala Gly Phe Val Gly Asn Lys Ala Val Val Gln Ala
        35                  40                  45

GCG GTT ACT ATT GCA GCT CAG AAT ACA ACA TCA GCC AAC TGG AGT CAG      192
Ala Val Thr Ile Ala Ala Gln Asn Thr Thr Ser Ala Asn Trp Ser Gln
    50                  55                  60

GAT CCT GGC TTT ACA GGG CCT GCT GTT GCT GCT GGT CAG AAA GTT GGT      240
Asp Pro Gly Phe Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val Gly
65                  70                  75                  80

ACT CTC AGC ATT ACT GCT ACT GGT CCA CAT AAC TCA GTA TCT ATT GCA      288
Thr Leu Ser Ile Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile Ala
                85                  90                  95

GGT AAA GGG GCT TCG GTA TCT GGT GGT GTA GCC ACT GTC CCG TTC GTT      336
Gly Lys Gly Ala Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val
            100                 105                 110

GAT GGA CAA GGA CAG CCT GTT TTC CGT GGG CGT ATT CAG GGA GCC AAT      384
Asp Gly Gln Gly Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn
        115                 120                 125

ATT AAT GAC CAA GCA AAT ACT GGA ATT GAC GGG CTT GCA GGT TGG CGA      432
Ile Asn Asp Gln Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg
    130                 135                 140

GTT GCC AGC TCT CAA GAA ACG CTA AAT GTC CCT GTC ACA ACC TTT GGT      480
Val Ala Ser Ser Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe Gly
145                 150                 155                 160

AAA TCG ACC CTG CCA GCA GGT ACT TTC ACT GCG ACC TTC TAC GTT CAG      528
Lys Ser Thr Leu Pro Ala Gly Thr Phe Thr Ala Thr Phe Tyr Val Gln
                165                 170                 175

CAG TAT CAA AAC TAACTCGAGC CC                                        552
Gln Tyr Gln Asn
            180
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 180 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro
 1               5                  10                  15
Arg Gly Ser His Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg
            20                  25                  30
Gly Trp Glu Phe Ala Gly Phe Val Gly Asn Lys Ala Val Val Gln Ala
        35                  40                  45
Ala Val Thr Ile Ala Ala Gln Asn Thr Thr Ser Ala Asn Trp Ser Gln
    50                  55                  60
Asp Pro Gly Phe Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val Gly
65                  70                  75                  80
Thr Leu Ser Ile Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile Ala
                85                  90                  95
Gly Lys Gly Ala Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val
            100                 105                 110
Asp Gly Gln Gly Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn
        115                 120                 125
Ile Asn Asp Gln Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg
    130                 135                 140
Val Ala Ser Ser Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe Gly
145                 150                 155                 160
Lys Ser Thr Leu Pro Ala Gly Thr Phe Thr Ala Thr Phe Tyr Val Gln
                165                 170                 175
Gln Tyr Gln Asn
            180
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 435 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA
(ix) FEATURE:

(A) NAME/KEY: Coding Sequence
(B) LOCATION: 1...432
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GCT GGC TTT GTT GGT AAC AAA GCA GTG GTT CAG GCA GCG GTT ACT ATT      48
Ala Gly Phe Val Gly Asn Lys Ala Val Val Gln Ala Ala Val Thr Ile
 1               5                  10                  15

GCA GCT CAG AAT ACA ACA TCA GCC AAC TGG AGT CAG GAT CCT GGC TTT      96
Ala Ala Gln Asn Thr Thr Ser Ala Asn Trp Ser Gln Asp Pro Gly Phe
            20                  25                  30

ACA GGG CCT GCT GTT GCT GCT GGT CAG AAA GTT GGT ACT CTC AGC ATT     144
Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val Gly Thr Leu Ser Ile
        35                  40                  45
```

```
ACT GCT ACT GGT CCA CAT AAC TCA GTA TCT ATT GCA GGT AAA GGG GCT      192
Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile Ala Gly Lys Gly Ala
    50                  55                  60

TCG GTA TCT GGT GGT GTA GCC ACT GTC CCG TTC GTT GAT GGA CAA GGA      240
Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val Asp Gly Gln Gly
65                  70                  75                  80

CAG CCT GTT TTC CGT GGG CGT ATT CAG GGA GCC AAT ATT AAT GAC CAA      288
Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn Ile Asn Asp Gln
                85                  90                  95

GCA AAT ACT GGA ATT GAC GGG CTT GCA GGT TGG CGA GTT GCC AGC TCT      336
Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg Val Ala Ser Ser
            100                 105                 110

CAA GAA ACG CTA AAT GTC CCT GTC ACA ACC TTT GGT AAA TCG ACC CTG      384
Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe Gly Lys Ser Thr Leu
        115                 120                 125

CCA GCA GGT ACT TTC ACT GCG ACC TTC TAC GTT CAG CAG TAT CAA AAC T    433
Pro Ala Gly Thr Phe Thr Ala Thr Phe Tyr Val Gln Gln Tyr Gln Asn
    130                 135                 140

AA                                                                   435
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ala Gly Phe Val Gly Asn Lys Ala Val Val Gln Ala Ala Val Thr Ile
 1               5                  10                  15
Ala Ala Gln Asn Thr Thr Ser Ala Asn Trp Ser Gln Asp Pro Gly Phe
            20                  25                  30
Thr Gly Pro Ala Val Ala Ala Gly Gln Lys Val Gly Thr Leu Ser Ile
        35                  40                  45
Thr Ala Thr Gly Pro His Asn Ser Val Ser Ile Ala Gly Lys Gly Ala
    50                  55                  60
Ser Val Ser Gly Gly Val Ala Thr Val Pro Phe Val Asp Gly Gln Gly
65                  70                  75                  80
Gln Pro Val Phe Arg Gly Arg Ile Gln Gly Ala Asn Ile Asn Asp Gln
                85                  90                  95
Ala Asn Thr Gly Ile Asp Gly Leu Ala Gly Trp Arg Val Ala Ser Ser
            100                 105                 110
Gln Glu Thr Leu Asn Val Pro Val Thr Thr Phe Gly Lys Ser Thr Leu
        115                 120                 125
Pro Ala Gly Thr Phe Thr Ala Thr Phe Tyr Val Gln Gln Tyr Gln Asn
    130                 135                 140
```

WE CLAIM:

1. A method for detecting anti- *Salmonella enteritidis* antibodies in animals, the method comprising:

reacting a sample obtained from an animal with a truncated Sef14 antigen under conditions to permit anti-Sef14 antibodies to bind the antigen, the truncated antigen having at least the native Sef14 signal peptide removed; and

correlating antibody-antigen binding with the presence of anti-SE antibodies in the sample.

2. A method for diagnosing *Salmonella enteritidis* infection in animals, the method comprising:

reacting a sample obtained from an animal with a truncated Sef14 antigen under conditions to permit anti-Sef14 antibodies to bind the antigen, the truncated antigen having at least the native Sef14 signal peptide removed; and

correlating antibody-antigen binding with *Salmonella enteritidis* infection.

3. The method of claim 1, wherein the truncated Sef14 antigen has the amino acid sequence of Sequence ID. No. 4.

4. The method of claim 1, wherein the truncated Sef14 antigen has the amino acid sequence of Sequence ID. No.6.

5. The method of claim 1, wherein said antigen is fixed to an inert surface prior to said reacting.

6. A Sef14 antigen consisting essentially of the amino acid sequence of Sequence I.D. No. 6.

7. An assay kit for the detection of anti-*Salmonella enteritidis* antibodies comprising an Sef14 antigen consisting essentially of the amino acid sequence of Sequence ID No. 6.

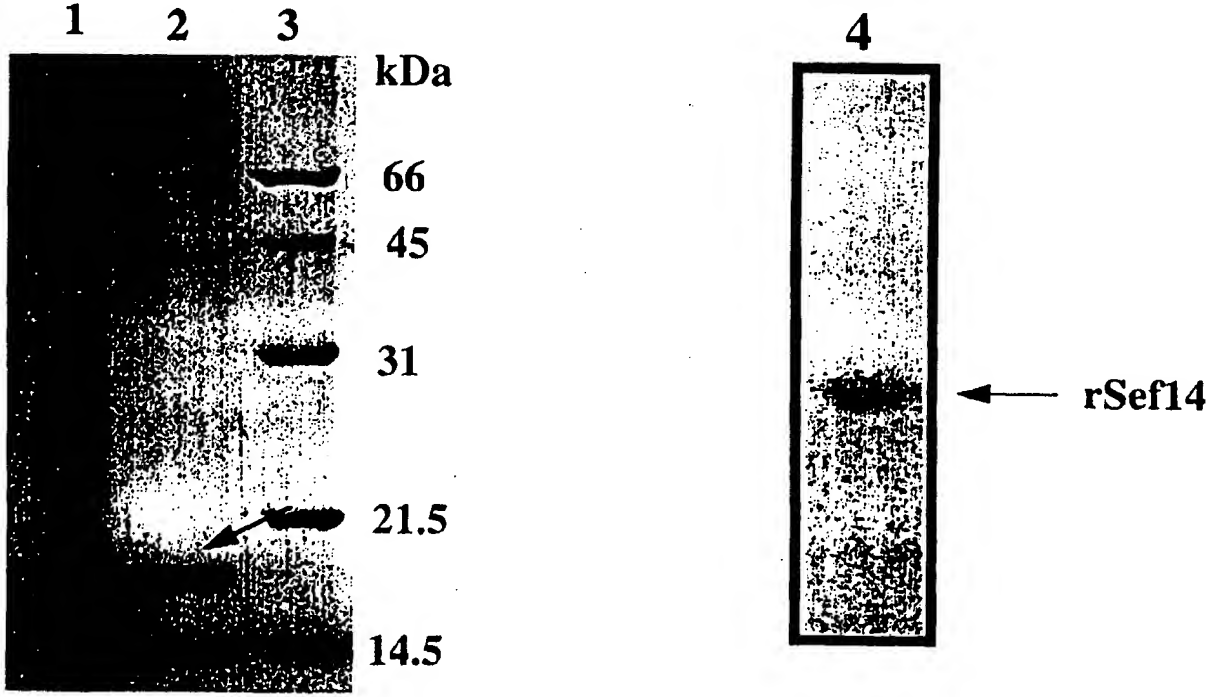
8. The assay kit of claim 7, wherein the antigen consists essentially of the amino acid sequence of Sequence ID. No. 4.

9. An antigen for stimulating the production of anti-*Salmonella enteritidis* antibodies comprising the amino acid sequence of Sequence ID No. 4 or 6.

10. The method of detecting anti-*Salmonella enteritidis* antibodies described in any of the foregoing claims, wherein the animal samples are obtained from fowl, and particularly from chickens or turkeys.

Figure 1

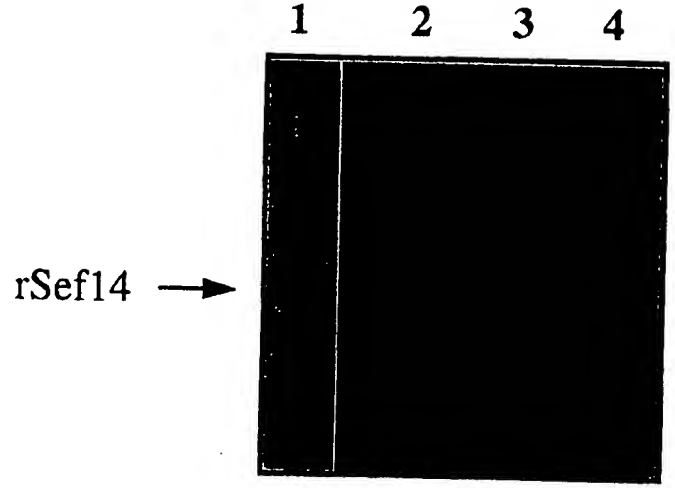
SDS-PAGE of rSef14 fragment protein



1. BL21DE3 (pET/*sefA*)before purification
2. Purified rSef14 fragment protein
3. LMW marker
4. rSef14 after electroelution

Figure 2

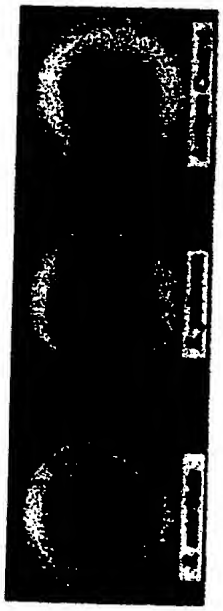
Western blot -- rSef14 fragment probed with Sef14 monospecific polyclonal antibody



1. Anti-Sef14
2. T7 tag antibody
3. BL21DE3 control
4. LMW marker

Figure 3

Latex agglutination test



A B C

Figure 4

1. Latex agglutination test.

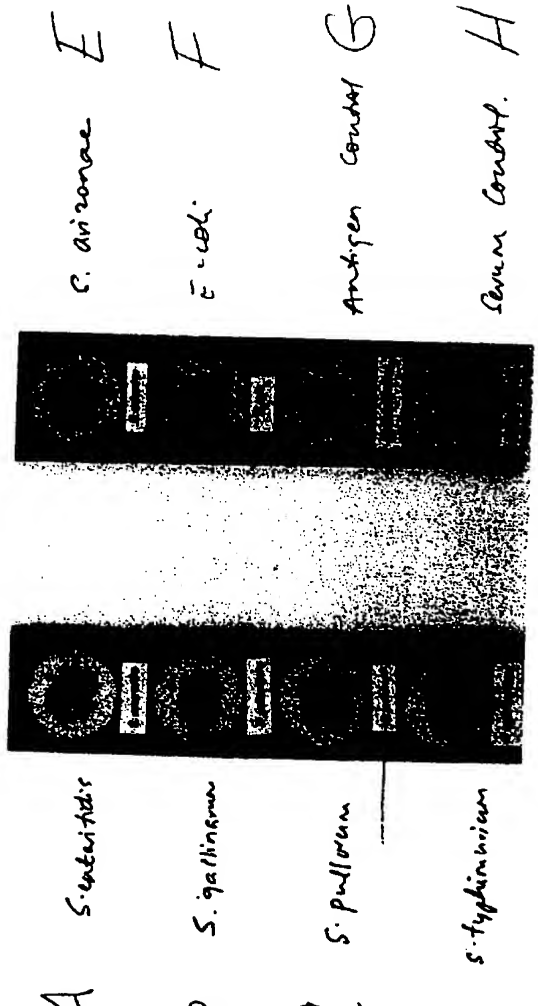


Figure 5

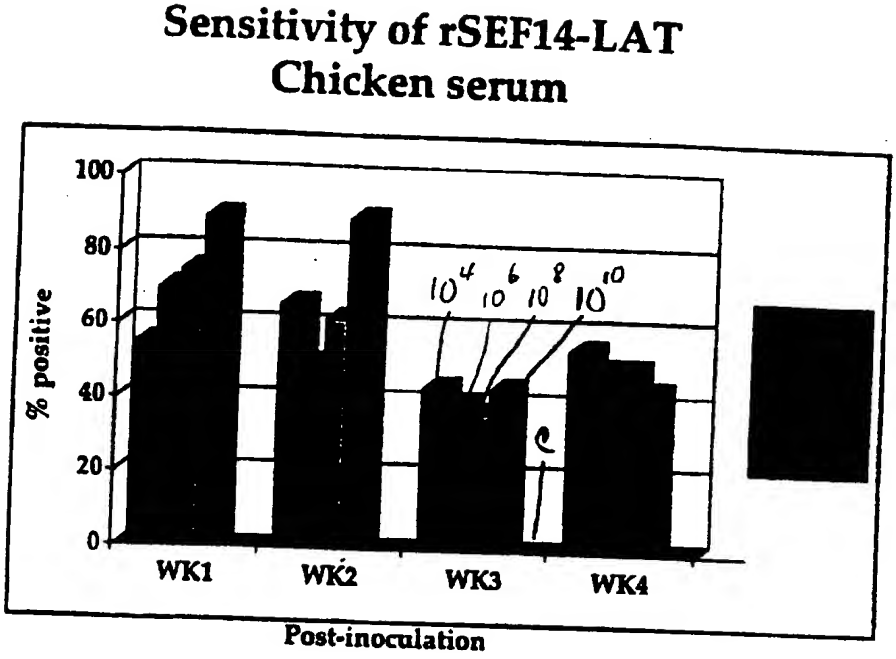

Sensitivity of rSEF14-LAT
Chicken serum
100
80
60
40
20
0
% positive
10^4 10^6 10^8 10^{10}
WK1
WK2
WK3
WK4
Post-inoculation

Figure 6

Sensitivity of SEF-14 ELISA Using Chicken Sera

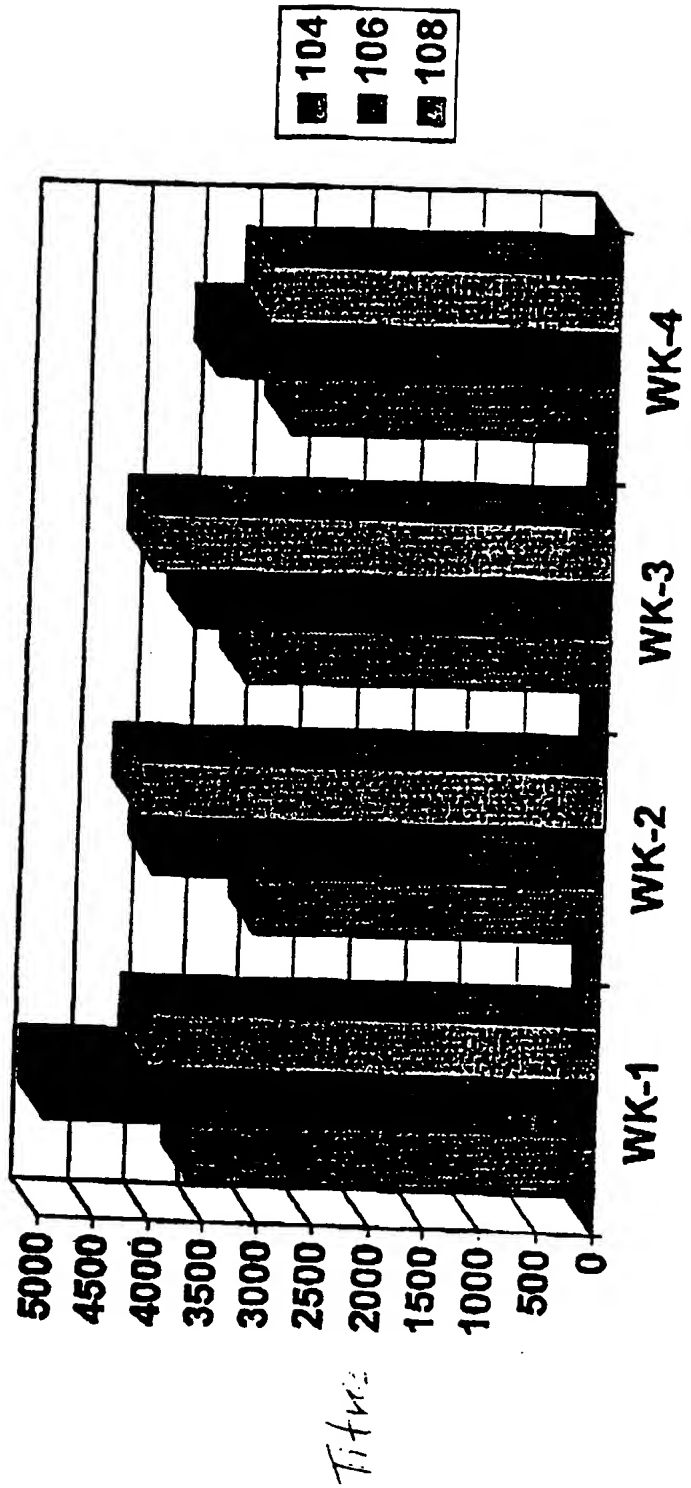
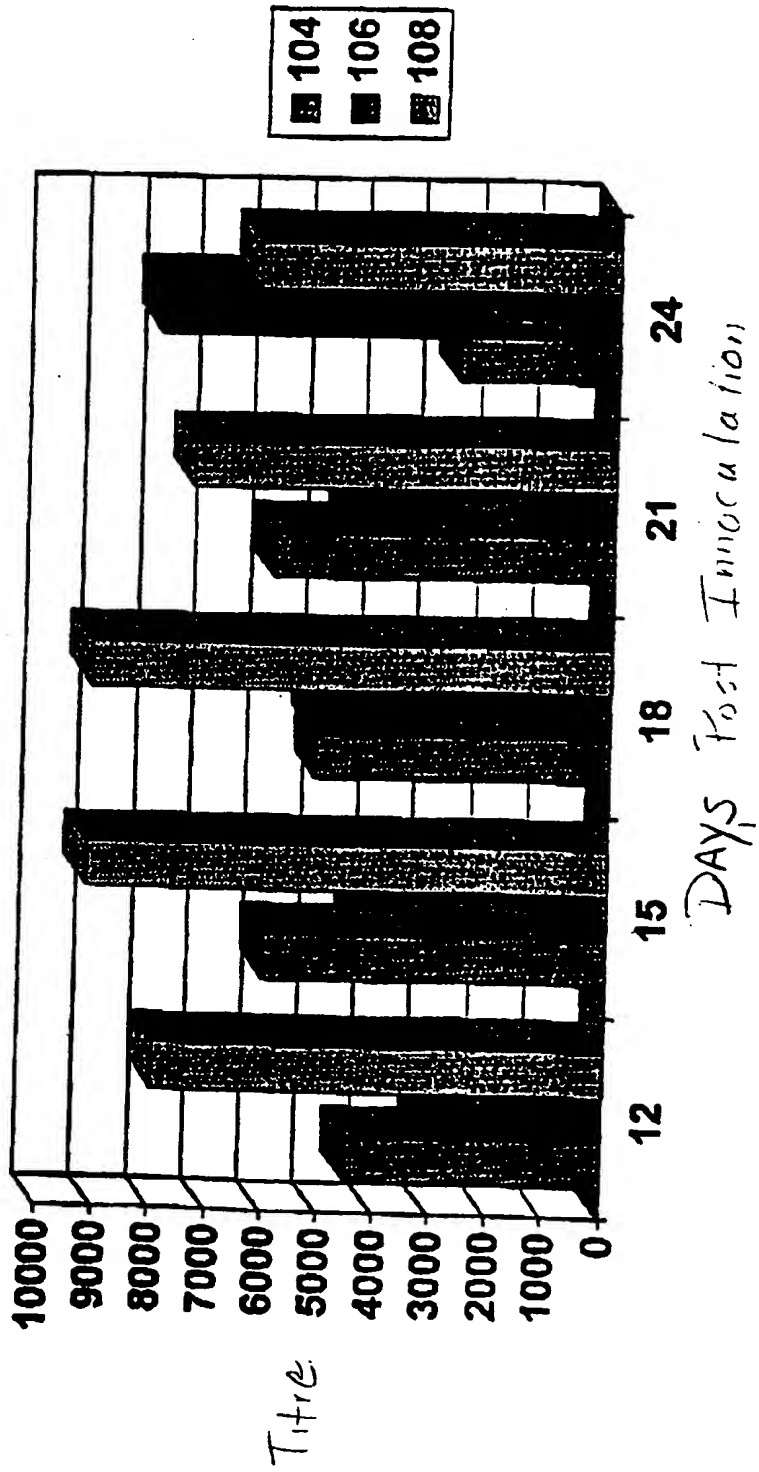


Figure 7

Sensitivity of SEF-14 ELISA Using Yolk



INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/US 97/12639

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/255 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THORNS CJ ET AL: "Development and application of enzyme-linked immunosorbent assay for specific detection of Salmonella enteritidis infections in chickens based on antibodies to SEF14 fimbrial antigen." J CLIN MICROBIOL, APR 1996, 34 (4) P792-7, UNITED STATES, XP002047275 see page 792 - page 793; table 1 ---	1,2,5,6, 10
X	WO 92 06197 A (MINI AGRICULTURE & FISHERIES) 16 April 1992 see claims 2,3 --- -/--	1,2,5,6, 10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
18 November 1997	02. 12. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/US 97/12639

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THORNS CJ ET AL: "The use of latex particle agglutination to specifically detect Salmonella enteritidis." INT J FOOD MICROBIOL, JAN 1994, 21 (1-2) P47-53, NETHERLANDS, XP002047276 see the whole document	1,2,5,6, 10
Y	WO 92 06198 A (MINI AGRICULTURE & FISHERIES) 16 April 1992 see claims 1-28	1,2,5,6, 10
Y	WO 93 20231 A (MINI AGRICULTURE & FISHERIES ;WOODWARD MARTIN JOHN (GB); THORNS CH) 14 October 1993 see claims 1-28	1,2,5,6, 10
A	CLOUTHIER SC ET AL: "Characterization of three fimbrial genes, sefABC, of Salmonella enteritidis." J BACTERIOL, MAY 1993, 175 (9) P2523-33, UNITED STATES, XP002047277	

1

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter ional Application No
PCT/US 97/12639

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9206197 A	16-04-92	AU 660152 B	15-06-95
		AU 8548991 A	28-04-92
		CA 2091982 A	02-04-92
		EP 0551325 A	21-07-93
		JP 6501934 T	03-03-94
		US 5510241 A	23-04-96
		AT 155169 T	15-07-97
		AU 660945 B	13-07-95
		AU 8656691 A	28-04-92
		CA 2091984 A	02-04-92
		DE 69126786 D	14-08-97
		EP 0551324 A	21-07-93
		WO 9206198 A	16-04-92
		JP 6502531 T	24-03-94
WO 9206198 A	16-04-92	AT 155169 T	15-07-97
		AU 660945 B	13-07-95
		AU 8656691 A	28-04-92
		CA 2091984 A	02-04-92
		DE 69126786 D	14-08-97
		EP 0551324 A	21-07-93
		JP 6502531 T	24-03-94
		AU 660152 B	15-06-95
		AU 8548991 A	28-04-92
		CA 2091982 A	02-04-92
		EP 0551325 A	21-07-93
		WO 9206197 A	16-04-92
		JP 6501934 T	03-03-94
		US 5510241 A	23-04-96
WO 9320231 A	14-10-93	AU 3895293 A	08-11-93